REMARKS

Applicant respectfully requests reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

Claims 33, 34, 37-40, 42 and 43 are requested to be cancelled. Applicants reserve the right to pursue the subject matter of the canceled claims in subsequent divisional applications. The cancellation of claims does not constitute acquiescence in the propriety of any rejection set forth by the Examiner.

Claims 32, 35, 36, and 41 are currently being amended.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

After amending the claims as set forth above, claims 32, 35, 36, 41 and 44 are now pending in this application.

Claim Objections

A. The Examiner objects to claim 32 because the term "populations" in line 5 should be in singular form. Applicants have amended claim 32 by replacing the term "populations" with "population." Applicants respectfully request reconsideration and withdrawal of the objection.

B. The Examiner objects to claim 42 as being of improper dependent form for failing to further limit the subject matter of a previous claim. Applicants have canceled claim 42, thus rendering the rejection of this claim moot.

35 U.S.C. 112, First Paragraph

A. Claims 32-34 are rejected by the Examiner for lack of written description and lack of enablement. The Examiner asserts that the original disclosure does not specify the terms "first marker" and "second marker". The Examiner further asserts that the specification fails to specify a cell population infected with a pathogenic organism. Applicants respectfully request reconsideration and withdrawal of the rejection.

Applicants respectfully disagree with the Examiner's assertion. However, to expedite prosecution, Applicants have canceled claims 33 and 34, thus rendering the rejection of these claims moot. Additionally, Applicants have amended claim 32 by replacing the terms "first marker" and "second marker" with the terms "first dye" and "second dye," respectively. Support for this amendment is found throughout the specification. Exemplary support is found on page 8, line 6, through page 9, line 22. Applicants reserve the right to pursue the deleted subject matter and the subject matter of the canceled claims in subsequent divisional applications.

B. Claims 32-36 and 39-43 are rejected by the Examiner for lack of written description and lack of enablement. The Examiner asserts that the terms "a first marker" and "a second marker" embrace a genus of molecules that could be used as markers for hybrid cell sorting and any method for sorting the markers. The Examiner also asserts that a person of ordinary skill in the art would not be able to sort hybrid cells without using fluorescent dye staining and metabolic selection. Applicants respectfully request reconsideration and withdrawal of the rejection.

Applicants respectfully disagree with the Examiner's assertion. However, to expedite prosecution, Applicants have canceled claims 33, 34, 37-40, 42 and 43, thus rendering the rejection of these claims moot, and have amended claims 35, 36 and 41 to depend only from claim 32. Additionally, Applicants have amended claim 32 by replacing the terms "first marker" and "second marker" with the terms "first dye" and "second dye," respectively, as discussed above. Applicants have also amended claim 32 to recite "(d) purifying the resultant hybrid cell population by fluorescence activated cell sorting." Applicants reserve the right to

pursue the deleted subject matter and the subject matter of the canceled claims in subsequent divisional applications.

C. Claims 32-44 are rejected by the Examiner for lack of enablement. The Examiner states that the specification fails to teach what the term "the *diversity* of the starting cell population is preserved in the resultant hybrid cell population" encompasses and how such diversity is preserved. Applicants respectfully request reconsideration and withdrawal of the rejection.

Applicants have canceled claims 33, 34, 37-40, 42 and 43, thus rendering the rejection of these claims moot. Additionally, Applicants have amended claims 35, 36 and 41 to depend only from claim 32.

With respect to the remaining claims, Applicants assert that a person of ordinary skill in the art would know what the term "the *diversity* of the starting cell population is preserved in the resultant hybrid cell population" encompasses. *See, e.g.*, Festuccia, C., Anticancer Research, 20(6B):4367-4371 (November 2000) (Exhibit 1), Dwarakanath, B., Indian Journal of Medical Research, 100:127-134 (September 1994) (Exhibit 2), and Gorunova, L., Genes, Chromosomes & Cancer, 23(2):81-99 (October 1998) (Exhibit 3), respectively, which relate to tumor diversity/heterogeneity. For example, Festuccia et al. is related to the heterogeneity of PC3 prostate cancer (see abstract). Festuccia et al. state that PC3 cell line contains different cell variations with different growth rates. For example, one variant grows as spherical multicellular aggregates and shows anchorage-independent growth. A second variant grows as single small rounds and shows anchorage-dependent growth without cell spreading. A third variant grows ad adherent cells. Dwarakanath et al. describes the intra-tumor heterogeneity/diversity of human brain tumors (see abstract). Additionally, Gorunova et al. discusses the heterogeneity/diversity of pancreatic carcinoma (see abstract). Additionally, Furthermore, the specification provides sufficient enablement for a person of ordinary skill in the art to preserve the diversity of the starting cell population in the resultant hybrid cell population. Therefore, a person of ordinary skill in the art would have known the meaning of the term "the diversity of the starting cell population."

The specification provides sufficient guidance to enable a person of ordinary skill in the art to preserve the diversity of the starting cell population in the resultant hybrid cell population. For example, Example 1 describes a method of forming hybrid cells in which the *diversity* of the starting cell population is preserved in the resultant hybrid cell population.

Additionally, attached is a Declaration Under 37 C.F.R. § 1.132 by Dr. T. Wagner which illustrates that the *diversity* of the starting cell population is preserved in the resultant hybrid cell population. The Declaration is discussed in detail below with respect to the Examiner's rejection of the claims as obvious over Gong et al. in view of Koolwijk et al.

35 U.S.C. § 112, Second Paragraph

Claims 32-44 are rejected by the Examiner as being indefinite.

A. The Examiner asserts that the metes and bounds of the claims are uncertain because the following concepts are unclear:

1. whether the first and second dye are the same or different;
2. the standards for cell sorting;
3. the correlation between the first and second dye of steps (a) and (b); and
4. the cell sorting process in step (d).

Applicants have canceled claims 33, 34, 37-40, 42 and 43, thus rendering the rejection of these claims moot, and have amended claims 35, 36 and 41 to depend only from claim 32. Applicants have amended claims 32 and 44 to address the issues raised by the Examiner. Therefore, claims 32, 35, 36, 41, and 44, as amended, comply with the requirements of 35 U.S.C. § 112, second paragraph.

B. The Examiner assets that the limitation "the diversity of the starting cell population is preserved in the resultant hybrid cell population" is indefinite because the specification fails to define the term. The meaning of the term "the diversity of the starting cell population is preserved in the resultant hybrid cell population" was within the knowledge of a person of ordinary skill in the art at the time of the present invention. As discussed above, Exhibits 1, 2 and 3 show that a person of ordinary skill in the art would be familiar

with the concept of tumor diversity/heterogeneity. Applicants respectfully disagree with the Examiner and request reconsideration and withdrawal of the rejection.

C. The Examiner asserts that in claim 33 there is insufficient antecedent basis for the limitation "said affected cells." Applicants have canceled claim 33, thus rendering the rejection of this claim moot.

D. The Examiner asserts that in claim 41 there is insufficient antecedent basis for the limitation "said pharmaceutically acceptable vehicle." Applicants have canceled claim 34, thus rendering the rejection of this claim moot.

35 U.S.C. § 103

Claims 32, 35-38, 41 and 44 are rejected under 35 U.S.C. § 103 as being unpatentable over Gong et al. in view of Koolwijk et al. The Examiner asserts that it would have been obvious to one of ordinary skill in the art at the time the invention was made to apply the methods taught by Koolwijk et al., in the process for selection and purification of dendritic-tumor cell hybrids as taught by Gong et al. with reasonable expectation of success. Applicants respectfully traverse and request reconsideration and withdrawal of the rejection.

The Examiner has failed to establish a *prima facie* case of obviousness because all three of the criteria required to establish a *prima facie* case of obviousness have not been met. In order to establish a prima facie case of obviousness, three basic criteria must be met. First, the prior art references (or references when combined) must teach or suggest all the claim limitations. Second, there must be a reasonable expectation of success. Finally, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. See MPEP 2142.

First, Gong et al. and Koolwijk et al. fail to teach or suggest each and every limitation of the present invention. As discussed previously, a feature of the present invention is that the heterogeneity/diversity of the starting cell population is preserved in the hybrid cells. With respect to the present claims, one of the cells in the hybrid cell (the "target" cell) is a

tumor cell and the second cell is a different type of cell, such as an antigen presenting cell. It is well known in the art that a tumor is often comprised of heterogeneous sub-populations of cells. Maintaining this heterogeneity in the population of hybrid cells that results from the fusion of the tumor cells with the second cells is important. If a vaccine is administered wherein only a fraction of the sub-populations of tumor cells are represented, an immune response will only be triggered against the represented types of tumor cells. The sub-populations of tumor cells not represented in the vaccine grow stronger and multiply faster and the efficacy of the vaccine is decreased. To prevent such an outcome, it is important to maintain the heterogeneity/diversity of tumor cells used as starting material in the population of hybrid cells used as a vaccine.

Classical selection methods, such as the method employed by Gong et al. involving the use of metabolic selection by culturing cells in HAT medium, fail to preserve the heterogeneity/diversity of starting material cells because some of the sub-populations of starting material cells are eliminated by the selection step. Because preservation of the heterogeneity/diversity of the starting cell population in the hybrid cells is a feature of the present invention, classical selection methods, such as the method employed by Gong et al., are not suitable for the present invention. Therefore, Gong et al. and Koolwijk et al. fail to teach or suggest all the claim limitations.

The Examiner refers to an excerpt in Gong et al. (right column, page 558) which states "the present studies have pursued an alternative DC-based strategy for inducing immunity against known or unidentifiable tumor antigens." The Examiner concludes that this statement indicates that Gong et al. teach that the heterogeneity/diversity of the starting cell population is preserved for a cancer vaccine. Applicants believe that the Examiner is misreading Gong et al. In context, the statement in the Gong et al. article clearly refers to the possibility of using his technique on tumor cells for which there is no characterized tumor antigen and is not a reference to preservation of population heterogeneity. This statement in Gong et al. would not cause a person of ordinary skill in the art to believe that the heterogeneity/diversity of the starting cell population of Gong et al. is preserved because, as discussed above, Gong et al. utilizes classical selection methods (fused cells were plated in the presence of HAT medium for 10-14 days – right column, page 560). Therefore, a person of ordinary skill in the art

would know that the method of Gong et al. does not maintain the diversity of the starting cell sub-populations.

The attached Declaration of Dr. Thomas E. Wagner Under 37 C.F.R. § 1.132 illustrates that hybrid cells are capable of retaining all antigens carried by the original population of tumor cells and that tumor antigen diversity of the original population of tumor cells is lost during long culturing periods. In Experiment I, B16F0/hIL-2 cells were stained with red dye and used in a PEG-mediated fusion with dendritic cells stained with green dye. Hybrid cells sorted out by FACS were cytospan on slides and immunocytochemically stained for the antigen (human IL-2). Figure A shows the isotype control. Figure B shows anti-human IL-2 antibody staining. The data presented in Figures A and B shows that all the hybridomas are human IL-2 positive. These results confirm that hybridomas are capable of retaining all antigens carried by the original population of tumor cells.

In Experiment II, B16F0/GFP tumor cells were either cultured alone or mixed with B16F0 tumor cells in a ratio of 1:1. The GFP positive cells were then monitored by fluorescent activated cell sorter (FACS) and plotted against time. Figure A is a graph showing culture of B16F0/GFP tumor cells over a 20 day period. Because there is no selective pressure for the GFP marker protein, the GFP marker protein disappears from the cultured cells over the time period observed. Figure B is a graph showing culture of a 1:1 ratio of B16F0/GFP tumor cells:B16F0 tumor cells over a 20 day period. Over time, the GFP marker protein is lost from cells and the culture is more homogeneous in cells lacking the GFP marker. The data presented in Figures A and B show that antigen is lost during culture and cells carrying particular antigens can be lost during culture. These results confirm the theory that tumor antigen diversity of the original population of tumor cells is lost during long culturing periods. For example, after 5-10 days of culturing, the population of cells expressing the GFP antigen decreased from 50% to about 20%. These results suggest that if GFP-expressing cells had comprised a minor portion of the cell population (e.g., 2%), after 5 to 10 days in culture, GFP-expressing cells would no longer comprise a detectible portion of the cell culture.

Second, a person of ordinary skill in the art would not have had a reasonable expectation of success in combining the teachings of Gong et al. and Koolwijk et al. to arrive at the present invention. A person of ordinary skill in the art would know that the method of Gong et al. does not maintain the diversity of the starting cell sub-populations. The Examiner is at best using an improper "obvious to try" standard. However, "'obvious to try' has long been held to not constitute obviousness." *In re Deuel*, 51 F.3d 1552, 1559, 34 USPQ2d 1210 (Fed. Cir. 1995).

Finally, there is no suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to combine the teachings of Gong et al. and Koolwijk et al. to arrive at the present invention. As discussed above, a person of ordinary skill in the art would know that the method of Gong et al. does not maintain the diversity of the starting cell sub-populations. Therefore, an ordinary skilled artisan would not have been motivated to combine the teachings of Gong et al. with the teachings of Koolwijk et al. to arrive at the present invention.

The Examiner asserts that the ordinary skilled artisan would have been motivated to modify the method of Gong et al. with the teachings of Kookwijk et al. because double fluorescent cell sorting requires fewer steps and less time for making and selecting hybrid cells. Applicants respectfully disagree with the Examiner's assertion. The Examiner's logic suggests that double fluorescent cell sorting is appropriate for any application in which selecting cells is desired. While cutting steps and saving time may be taken into consideration by an ordinary skilled artisan when determining what method to use to achieve a goal, it is by no means the driving force. Instead, a person of ordinary skill in the art would chose the method that achieved the most accurate results. Once again, the Examiner is at best erroneously using an "obvious to try" standard. As discussed above, "'obvious to try' has long been held to not constitute obviousness." *In re Deuel*, 51 F.3d 1552, 1559, 34 USPQ2d 1210 (Fed. Cir. 1995).

CONCLUSION

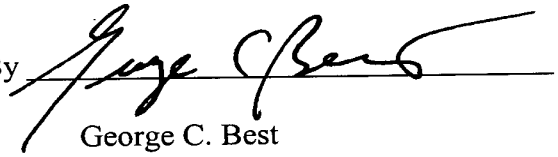
Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date October 3, 2003

By 

George C. Best
Attorney for Applicant
Registration No. 42,322

FOLEY & LARDNER
Customer Number: 22428
Telephone: (202) 945-6078
Facsimile: (202) 672-5399

ANTICANCER RESEARCH *20:* 4367-4372 (2000)

Culture Conditions Modulate Cell Phenotype and Cause Selection of Subpopulations in PC3 Prostate Cancer Cell Line

CLAUDIO FESTUCCIA, GIOVANNI LUCA GRAVINA, ADRIANO ANGELUCCI, DANILO MILLIMAGGI and MAURO BOLOGNA

Department of Experimental Medicine, Medical School, University of L'Aquila, 67100 L'Aquila, Italy

Abstract. *PC3 cell line contains different cell variants. A first variant grows as spherical multicellular aggregates and shows anchorage-independent growth. A second variant grows as single small rounds and shows anchorage-dependent growth without cell spreading. A third variant, representing the most abundant population, grows as adherent cells. These populations differ in α2β1 and α3β1 integrin expression with low levels in the suspended (S) cells, intermediate in partially adherent (R) cells and high in adherent cells (A). TPA, which up-regulates the expression of β1 integrins, increases invasiveness of cells. In addition, PC3 variants differ in MMP9 and uPA secretion and activity. High levels of TIMP1 and PAI1 present in S variant reduce MMP9 and uPA activities, respectively. In conclusion, PC3 cell line shows variants with strong phenotypic heterogeneity reflecting also the in vitro culture condition. Our observations may explain some of the contradictions in the literature. Therefore, the data obtained with this line should be evaluated more carefully, considering morphological and functional characteristics of the possible variants in the cell population. However, this heterogeneity may represent a good model in the study of tumor progression.*

A metastatic event requires several genetic and epigenetic changes in a multistep process determining a selection of malignant cell subpopulations. Transition from *in situ* tumors to metastatic diseases is defined by the ability of cells to invade local tissue and to cross tissue barriers. Interactions with the extracellular matrices (ECM), and basement membranes (BM) include attachment, proteolysis and migration through the matrix defects (1). Several cell surface adhesion molecules have been identified, each with different function. Integrins are a large class of α/β heterodimeric receptors that mediate cellular adherence modulating cell morphology, locomotor capacity, and state of differentiation. It is therefore not surprising that the presence or absence of several of these molecules has been demostrated to correlate with metastatic potential (2). Despite the existence of several modern strategies in the clinical management of prostatic carcinoma (PRCA), little progress has been made to predict the behaviour of the individual tumor. Biological studies are limited by the lack of adequate *in vitro* models and of suitable cell lines. The PC3 cell line represents a widely used model in the study of metastasis. However, contradictory experimental data are available in the literature. In fact, PC3 cells contain several cell types, reflecting the phenotypic heterogeneity of the cell lines. Kaighn and coworkers (3) observed that these cells can grow as epithelial-like monolayers with occasional spherical aggregates suggesting the possibility of isolating clonal variants. Our study is focused on the possibility that morphologic and functional changes within the PC3 cell line may heavily influence experimental data. This may be due to cell heterogeneity promoted by culture conditions which can cause the selection of different cellular variants. We have characterized the ECM-adhesive characteristic and the proteolytic and invasive profiles of three different population variants obtained from PC3.

Correspondence to: Claudio Festuccia, Dipartimento di Medicina Sperimentale, Cattedra di Patologia Generale, Università dell'Aquila, Via Vetoio, Coppito 2, 67100 L'Aquila, Italia. Fax: (39) (0862) 433523, e-mail: festucci@univaq.it

Key Words: Prostate cancer, cell culture, cell selection.

Materials and Methods

Cell culture and reagents. Human prostatic cancer cell line PC3 was purchased from the ATCC (Rockville, MD, USA) and grown in DMEM supplemented with 10% foetal calf serum. All reagents, unless otherwise specified, were purchased from Sigma (St. Louis, MO, USA). Monoclonal antibodies against α1, α2, α3, α4 (clone P4G9), α5 (clone P1D6), αvβ5 (clones P4AC10, P4G11 and P5D2), β2 (clone P4H9), β3 and β4 (clone 3E1) and polyclonal antibodies against α6 integrins subunit; were kindly provided by Dr. J. Marshall (ICRF, London UK). Matrigel was a kind gift of Dr. A. Albini (Advanced Biotechnologic Centre Genova, Italy). Plastic ware was obtained from Nunc (Roskilde, Denmark). Tissue culture media and reagents were obtained from Hyclone (Oud-Beijerland, Holland).

FACS analysis. Quantification of positive cells was performed by flow cytometric analysis (FACScan; Becton Dickinson, Mountain View, CA, USA). Membrane integrin expression was evaluated on PC3 variants by FACS using specific Abs: a FITC-labelled secondary antibody was used.

Zymographic analysis. Zymography was performed using SDS-polyacrylamide gels copolymerized with 0.1 mg/ml gelatin type B for gelatinase expression and 0.1 mg/ml lactose-free casein and 100 μg/ml

human plasminogen for plasminogen activator expression as previously described (4, 5). The reverse zymographies for TIMPs and PAIs were performed as previously described (4, 5).

Adhesion and invasion assays. Plates (24-wells) were coated with 50 μg/ml of polylysine, Matrigel, collagens type I and IV and laminin in order to verify the pattern of cell adhesion. Cells were plated at 5×10^5 cells/well and adhesion verified in a time-course experiment. A detailed description of the procedure is published elsewhere (6). The ability of the cells to migrate across a Matrigel barrier was determined using 50 μg/ml Matrigel by a modified chamber method (7).

Results

Morphological characteristics of PC3 subpopulations. The morphology of PC3 cells, as seen by phase contrast microscopy, is showed in Figure 1. PC3 cells were grown primarily as monolayer of flat cells with large cytoplasm and wide nuclei with prominent nucleoli. When cultured at high density, some cells formed clusters of suspended cells which furthermore retained the capability to adhere. Then, it was possible to select cells with low capability to adhere to different substrates. A third population of cells presented adhesive capabilities without cellular spreading. These cells adhered with a small membrane portion forming a monolayer of rounded cells with high nucleus cytosplasm ratio.

Determination of membrane integrin profiles using FACS analysis and function experiments. PC3 subpopulations were analyzed by flow cytometry to determine integrin expression. Figure 2 shows that the levels of β1 were high in all three cell variants. The levels of α2 and α3 integrin subunits were high in PC3A, moderate in PC3R and very low in PC3S. PC3A variant presented also high levels of α5 and αv. All cells lacked expression of β4. To assess the functional role of α2β1 and α3β1 in binding of PC3 cells to type I and IV collagen, laminin and Matrigel, we used blocking antibodies. Anti-α2, anti-α3 and anti-β1 antibodies inhibited, in a dose-dependent manner, the adhesion of PC3 cells to collagens. Adhesion of PC3A was inhibited 60%, 40% and 50% by 10 μg/ml antibodies against α2, α3 and β1, respectively (Figure 3) suggesting that α2,B1 and α3β1 act as receptors for type I and type IV collagen in these cells. The anti-α2 Ab blocked totally adhesion of PC3R to type I collagen, whereas the inhibition was very low with anti-α3 Ab and adesion to type IV collagen, was not affected.

As shown in Figure 4A, PC3 cells migrate toward a Matrigel-coated filters. PC3A and PC3R migrated more than PC3S. Function-blocking experiments (Figure 4B) showed that β1 specific Abs inhibited the migration on Matrigel more effectively in PC3R respect PC3A due to the lower levels of β1 in PC3R. This figure also suggests that the receptor for laminin on PC3R may be primarily α6β1 and not α3β1 or α2β1 since that anti α6 Ab inhibited the attachment of these cells to laminin and to Matrigel whereas the anti-α3 Ab or anti-α2 Ab did not (or only partially).

Adhesion to collagen type I and IV was induced in PC3S

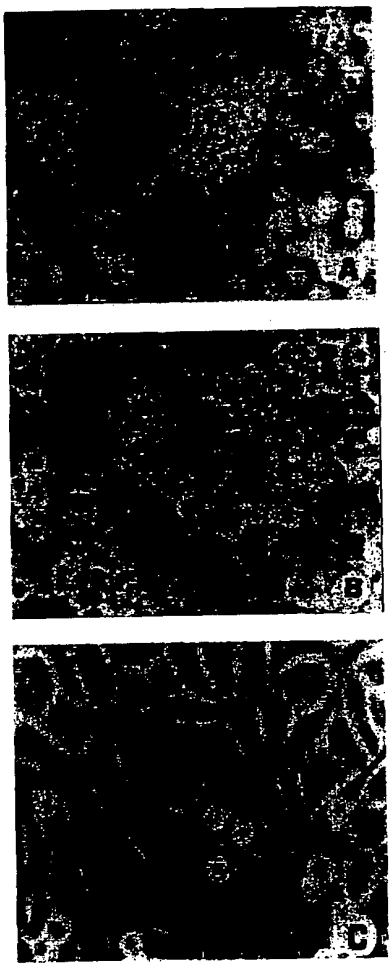


Figure 1. *Morphology of PC3 cell variants at 400X:*
A. PC3S variant with anchorage-independent growth;
B. PC3R variant with anchorage-dependent growth showing rounded morphology and a foot-like adhesion area;
C. PC3A variant with anchorage-dependent growth showing flat morphology and tight adhesion.

and PC3R variants by treatment with TPA as shown in Figure 5 and this was explained in the increased expression of integrins. In fact, TPA was also able to stimulate expression of α2β1 and α3β1 (data not shown).

Protease expression. PC3 variants differed also for the protease and tissue inhibitor expression as shown in (Figure 6). This can explain some of the differences observed in the invasive capabilities. In fact, the high levels of TIMP1 and PAI1 observed in the S variant suggests a down-regulation of uPA activity, a limitate activation of MMP9 by plasmin in high levels of plasminogen and in serum free conditions in these cells.

Discussion

Adhesive interactions are now thought to transduce signals to intracellular cytoskeletal proteins modulating movement and

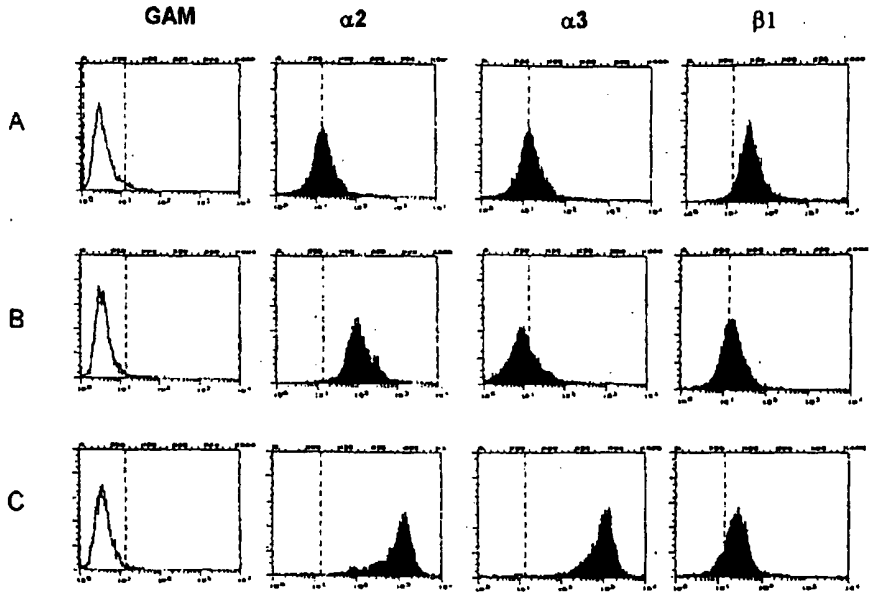


Figure 2. *FACS analysis of PC3S (A), PC3R (B) and PC3A (C) cell variants.*

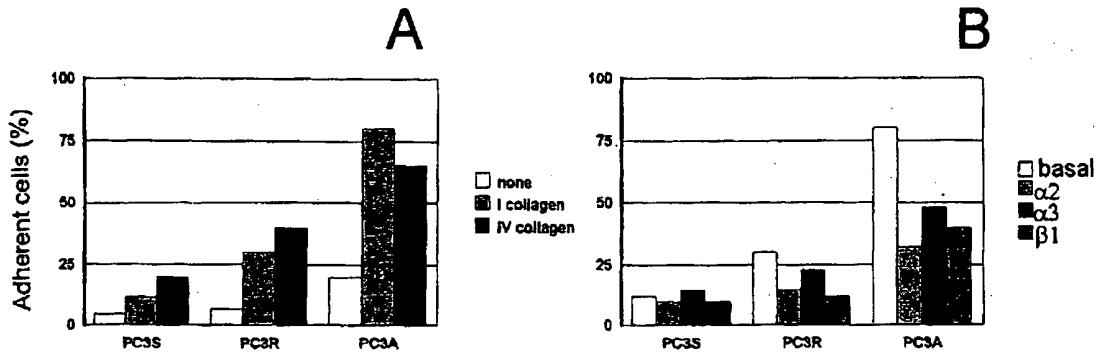


Figure 3. *Adhesion of PC3 cell variants on type I and type IV collagen- coated plates (A) and effect of blocking mAbs (10 μg/ml) against α2, α3 and β1 integrins (B).*

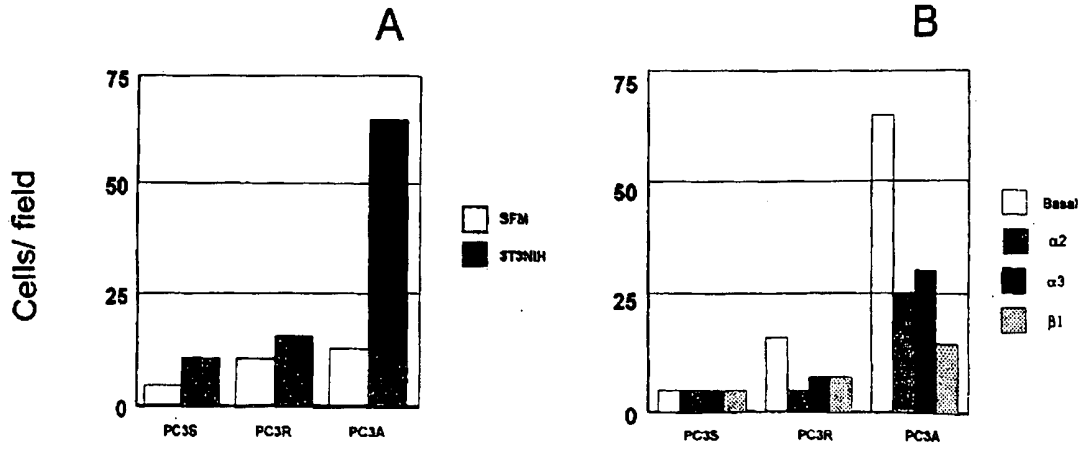


Figure 4. *Matrigel invasion of PC3 cell variant (A) and effects of blocking mAbs (10 μg/ml) against α2, α3 and β1 integrins (B).*

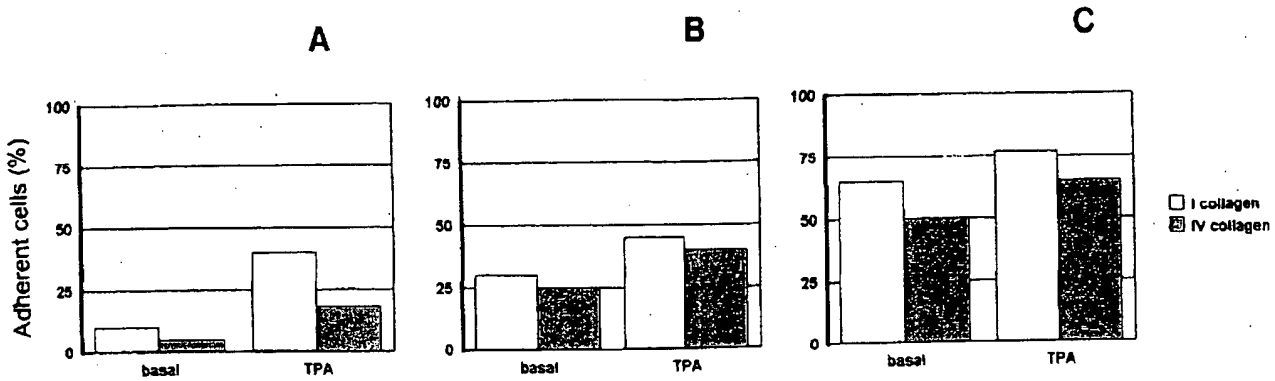


Figure 5. *Role of 100 ng/ml of TPA in inducing adhesion in PC3S (A), PC3R(B) and PC3A (C) cell variants.*

altering gene expression (8). Currently, the molecular mechanisms involved in these processes are largely not completely elucidated, although it is likely that reorientation of integrin cytoplasmic domains induced by ligand binding start the signalling responses that include transduction of mechanical forces to the cytoskeleton (9) and spatial compartimentalization of signalling complex (10). In this paper we studied the role of integrins in modulating tumor cell adhesion migration and protease expression in variants of PC3 human prostatic tumor cell line. Recent studies have examined the role of tumor cell attachment via β1 integrin receptors. Anti β1 MoAb inhibits both tumor cell migration and *in vivo* invasiveness. Our data showed that adhesion to type I and IV collagens is blocked by Abs against α2, α3 and β1 integrin subunits. Overexpression of α2 and α3 integrins increases cell adhesion, induces the formation of metastatic colonies in nude mice and also correlates with tumor progression, since it is expressed at high levels in metastatic cancers (11, 12). In our experiments α2 and α3 integrins are up-regulated only in PC3A variant. In addition, although we observe basal low levels of β1 expression in PC3R and PC3S variants, we notice that growth and motility factors may up-regulate β1 expression in these cells as well as in PC3A (6). This may be explained by hypothesizing that tumor cells may become more agressive during the invasive process by interaction between metastatic cells and host environments (ECM components and soluble factors) through modulation of integrin expression and protease activity. In fact, cyclic attachment to ECM components and subsequent release of protelytic enzymes occurs in a directed and controlled manner. In prostate cancer several studies have demostrated that the uPA/plasmin system plays a significant role in tumor cell invasion and metastasis (4, 13-15) and gave clues pointing to a possible mechanism causing concentration of uPA at the plasma membrane site where the cell establishes a contact with the substratum (16, 17). TPA (15), as well as growth factors like TGF-β1 (18) and Gastrin-Related Peptides/Bombesin (19), are able to induce uPA and MMP9

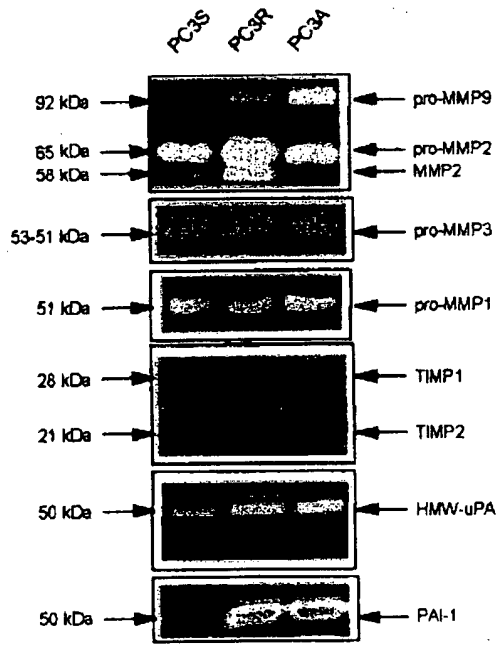


Figure 6. *Protease and tissue inhibitors secreted by PC3 cell variants. Differences were observed in MMP9 and uPA expression (higher levels in PC3A), MMP2 activation (higher levels in PC3R), TIMP1 and PAI-1 (higher levels in PC3S). No significant differences in the expression of stromelysin 1 (pro-MMP3), collagenase 1 (pro-MMP1) and TIMP2.*

secretion in PC3 cells and also to cause changes in the cell shape and integrin expression, primarily involving α2β1 and α3β1 (ms in preparation).

Prostate cancer cells produce high levels of MMP9 and MMP2. Nevertheless only MMP9 can be related to the invasive phenotype (5, 20, 21). In our experiments we demostrated high levels of MMP9 in PC3A, whereas pro- and active forms of MMP2 are higher in PC3R variant. This is in agreement with Teti *et al.* (22) who found that the expression

and the activation of MMP-2 can be modulated by the cell shape. Indeed, cell variants growing as spheroid aggregates (in agar or in microgravity conditions) produced higher levels of MMP-2 respect to the variants of the same line grown in monolayer (23). The uPA production was similar in all variants. Anyway plasmin activity was lower in PC3S (data not shown) because of high levels of PAI1. In addition PC3S cells secrete high levels of TIMP1. Taken together these results indicate that although the amounts of proteases are evident, the net proteolytic activity in this subpopulation is very low. Therefore, PC3S variant shows elevated cell proliferation activity and higher responsiveness to growth factor's like EGF (data not shown). These results suggest that the PC3S variant may be considered an actively proliferating cell population with low basal but inducible invasive capacity by which can originate more metastatic cells. In conclusion, the PC3 cell line showed a strong phenotypical heterogeneity (which is definitely higher with respect to other prostatic cancer cell lines) reflecting the probabilistic variations of the *in vitro* culture conditions. These observations may indeed explain the contradictory results present in the numerous experiments with this cell line. Therefore, the results obtained with PC3 cells should be analyzed more carefully, in view of the possibility that different specific cell variants may have been selected in different laboratories. However, the cellular heterogeneity could cause a more selective plasticity to proliferative /inhibitory stimula and the selection of cell variants from the same cell lines could represent a suitable model to study the tumor progression.

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Heterogeneity in DNA content & proliferative status of human brain tumours

B.S. Dwarakanath*, P.S. Manogaran**, Sarala Das§, B.S. Das§§ & Viney Jain*

Departments of Biophysics, §Neuropathology & §§Neurosurgery, National Institute of Mental Health & Neuro Sciences, Bangalore

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Intra-tumour and inter-tumour heterogeneity in the cytokinetic organization was studied in 235 primary human brain tumours. DNA index (DI; relative tumour cell DNA content) and proliferating fraction (%PF; a measure of proliferative status) were analyzed in tumour biopsy by flow cytometry using a DNA specific fluorochrome (DAPI) and internal standards (chicken erythrocytes, CE). Incidence of micronuclei was studied in tumour biopsy tissue as well as in explants maintained in organ culture. Clonal diversity (implied by the presence of multiple peaks in the DNA histograms) was highest among medulloblastomas (44%) followed by gliomas (19%) and meningiomas (14%). Nearly 85 per cent of the malignant gliomas analyzed (histological grade III/IV) exhibited a great deal of regional variation in the proliferative status as well as micronuclei frequency as compared to meningiomas. Inter-tumoural variations in the DNA content was highest among gliomas ($0.9 < DI < 3.6$) and lowest among schwannomas ($1.7 < DI < 2.2$). Similarly, the distribution of %PF values was also broader (10 - 49%) in gliomas as compared to the other primary brain tumours (5 - 36%). Analysis of tumours taking both DI and %PF values improved the ability to discern histologically graded low and high tumours. Analysis of clonal diversity and spatial heterogeneity in the cytokinetic parameters could complement the clinicopathological findings in assessing the biological behaviour of human brain tumours, facilitating the prognostification and design of optimal treatment regimen.

Key words Brain tumours-DNA index-micronuclei-proliferative status-tumour heterogeneity

Human cerebral gliomas which constitute about 60 per cent of the central nervous system (CNS) neoplasms are refractory to currently available therapeutic measures. This is attributed mainly to the heterogeneity in their biological behaviour[1,2]. Inter- and intra-tumoural heterogeneity in morphology, DNA content, karyotype, proliferative capacity, doubling time, colony forming ability, antigenic expression, resistance to cytotoxic drugs and radiation, repair of DNA damage *etc.*, have often been observed[2-6]. Since the tumours of the CNS arise in the cranium and have limited space to grow before becoming symptomatic, it has been suggested that, the growth rate may directly affect the prognosis[7]. Therefore, qualitative as well as quantitative analysis of proliferation potential and other parameters reflecting the biological behaviour of individual tumours may profoundly influence their management.

Deviation from the diploid DNA content, one of the markers of malignancy[8], together with proliferative activity may reflect the biological behaviour and hence influence the prognosis[9-11]. Clinical studies have shown that the response to chemotherapy of children harbouring a mixed population of diploid

Present addresses: * Department of Biocybernetics, Institute of Nuclear Medicine and Allied Sciences, Delhi 110054
** Center for Cellular and Molecular Biology, Hyderabad

and non-diploid clones was different than children harbouring tumours with only a diploid clone in case of neuroblastoma[12] and medulloblastoma[13]. Spontaneous appearance of micronuclei in a cell population indicates a higher degree of chromosomal instability, proliferative activity and cell loss, that is generally associated with malignant changes[13]. Further, the micronuclei assay is rapid and easy to perform. Therefore, analysis of heterogeneity in the DNA content, proliferative activity, and micronuclei frequency may supplement histopathological findings and facilitate the design of treatment regimen for individual patients[14,15].

Flow cytometric measurements of cellular DNA content, have revealed a widely varying range of results in human brain tumours[16-19]. However, little effort has been made in qualitative as well as quantitative analysis of spatial heterogeneity and clonal diversity within a tumour besides variation among tumours of the same type. Therefore, the present investigations were undertaken to examine the intra-tumour and inter-tumour heterogeneity in the cytokinetic organization of human brain tumours using flow cytometric analysis of DNA content and proliferative characteristics as well as spontaneous expression of micronuclei.

Material & Methods

Tumours and normal brain tissue : A total of 235 primary brain tumours, comprising 110 glioma, 47 meningioma, 24 schwannoma, 19 medulloblastoma, 7 craniopharyngioma and 28 others (neurofibroma, ependymoma, haemangiopericytoma, tuberculoma *etc.*) included in this study (Table I), were analyzed between 1983 and 1988 at the National Institute of Mental Health and Neuro Sciences, Bangalore. Normal brain tissue was obtained from autopsies conducted within few hours of death,-in case of head injuries.

Flow cytometry : Tumour specimens provided by the Neurosurgery department were collected in cold minimum essential medium (MEM 199). The tissue was randomly divided into groups (up to 5 depending on the sample available) and freed of blood and fibrous material, by teasing and washing with the medium. Single cell suspensions were prepared by

Table I. Inter-tumour variations in various parameters derived from flow cytometric measurements of DNA content in human brain tumours

Tumour type (n)	DNA index Mean±SD (Range)	Diploid tumours[1] (%)	Aneuploid tumours[2] (%)	Multiploid tumours (%)	Tetraploid clone[3] (%)
Glioma (110)	2.00 ± 0.39 (0.90 - 3.60)	60	21	19	19
Meningioma (47)	1.85 ± 0.31 (0.65 - 2.67)	51	35	14	15
Schwannoma (24)	1.93 ± 0.12 (1.65 - 2.20)	88	12	0	8
Medullo-blastoma (19)	2.01 ± 0.48 (1.35 - 3.17)	38	18	44	22
Craniopharyngioma (7)	1.91 ± 0.15 (1.65 - 2.03)	67	33	0	0
Others[4] (28)	1.92 ± 0.19 (1.60 - 2.39)	65	28	7	0

1: 1.8 < DI < 2.2
2: DI > 2.2 or DI < 1.8
3: Tumours having a large G_2/M peak but without a significant S-fraction
4: Ependymoma, neurofibroma, haemangioblastoma, tuberculoma *etc.*
DI, DNA index

mechanical disruption using iris scissors and scalpel, followed by pipetting with pasteur and fine bore pipets. The preparation was filtered through a nylon mesh (150 μm) and washed with cold Hank's balanced salt solution (HBSS). The cells were fixed in 70 per cent chilled ethanol and stored at 4°C. Cells were stained with a DNA specific fluorochrome diamidino-2-phenylindole-dihydrochloride, DAPI (Serva, Heidelberg) as described earlier[20]. Briefly, cells washed once with distilled water were treated for 20 min with 0.22 *M* citric acid containing 0.5 per cent tween 20 detergent (*p*H 2.1) and subsequently stained with DAPI (5 m*M*), present in 0.4 *M* sodium phosphate buffer (*p*H 8.9).

Measurements were made on an ICP-22 flow cytometer (PHYWE, FRG), interfaced with multichannel analyzer (Canberra, 8100, USA). The flow rate was controlled using a syringe pump (Orion Inc., Massachusettes). Chicken erythrocytes (CE) stained identically and measured along with the samples served as internal standards[21], while, human peripheral blood leukocytes (PBL) served as reference standards, facilitating a precise estimation of the DNA content of various tumour samples. To compare the DNA contents of tumour samples, DNA index (DI) was calculated as follows[22]:

$$\text{DNA index} = \text{(DNA content of the tumour sample/DNA content of the reference std.) X 2}$$
$$= (P_S/P_{CE} + P_{PBL}/P_{CE}) \text{ X } 2$$

where, P_S, P_{CE} and P_{PBL} are the mean channel numbers of the tumour sample, chicken erythrocytes and human PBL respectively. To avoid the interference from multiplets of CE in the evaluation of cell cycle distribution (due to the overlapping with sample peaks), samples were also measured without the addition of CE.

Cell cycle distributions were evaluated by graphical methods, assuming a symmetrical Gaussian distribution of values around the G_1 (diploid) and G_2 (tetraploid) peaks. Percentage of cells in S-phase (S-fraction) and the fraction of cells in the S, G_2 and M phases added together, called the proliferating fraction (%PF) was calculated to compare the proliferative state of the tumours.

Micronuclei analysis : Incidence of cells with micronucei in freshly collected tumour biopsy tissue as well as in the primary explants maintained in organ cultures, were examined. The detailed procedure for maintenance of cultures and analysis of micronuclei has been published[23]. Percentage of cells with micronuclei called the M-fraction (MF %) was calculated as :

$$MF\ \% = N_m / N_t \times 100,$$

where N_m is the number of cells with micronuclei and N_t is the total number of cells analyzed.

Histology : Histological diagnosis and grading of tumours was done according to the WHO criteria[24], using H & E stained paraffin sections.

Quantitative analysis of the intra-tumoural heterogeneity in the %PF as well as M-fraction values was carried out by calculating the variance σ^2 (where σ is the standard deviation) of the respective mean values. The inter-tumoural heterogeneity was analyzed by calculating the mean values of %PF and S-fraction together with the standard deviation (SD).

Results

Histograms of cellular DNA content from normal brain tissue (seven areas of five brains) was characterized by the presence of a large G_1 peak (≈ 90% cells; Fig. 1a) representing the diploid DNA containing cells (DNA index = 2) and a tetraploid peak with ≈ 8 per cent of cells. Less than 2 per cent of the cells were seen in the region representating S-phase. On the other hand, a significant fraction of cells (≥ 15%) in the S-phase was observed in most tumours (Fig. 1b), indicative of cell proliferation. In nearly 15 per cent of the cases, multiple G_1, peaks, were seen implying the presence of more than one clonal population (Fig. 1c). In these tumours the evaluation of cell cycle distribution was hampered by the overlapping of peaks from different clones.

Variations in the clonal composition as well as regional differences in the cytokinetic organization were investigated. The heteroploid nature of the tumour was indicated by the presence of multiple G_0/G_1 peaks (Fig. 1c). Under the present conditions (CV value between 0.9 and 1.2 for human PBL) cell populations differing in their DNA content by ≥ 5 per cent could be clearly distinguished. The incidence of heteroploidy (Table I) was highest among medulloblastomas (44%), followed by gliomas (19%)

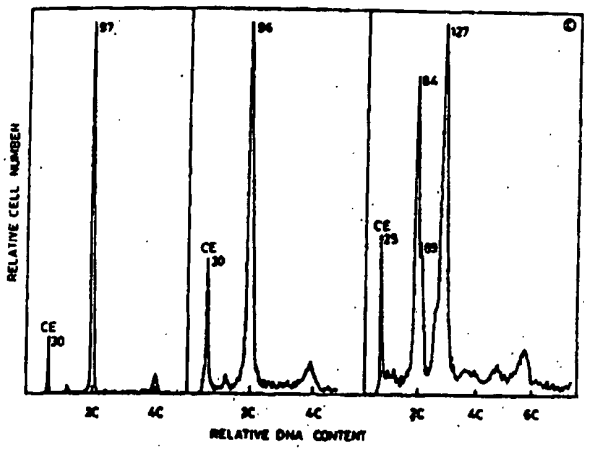

Fig. 1. DNA histograms obtained for cells from (a) normal brain tissue, (b) grade II astrocytoma and (c) grade IV astrocytoma, stained and measured along with chicken erythrocytes (CE) used as internal standards. Figures at the top of the peak are the relative channel numbers proportional to the DNA content. 2C represents the diploid DNA containing cells.

and meningioma (14%). While, 86 per cent of the tetraploid gliomas were found to be histologically malignant (AC III/IV or GM), 66 per cent of the meningiomas with multiple DNA indices showed features of histological malignancy. In nearly 15 per cent of the tumours (Table I), presence of a large second peak was observed at the position where the G_2 + M fraction of the first peak population would be expected, but with an insignificant fraction of S-phase cells. These tumours were considered to comprise distinct clonogenic populations with different DNA contents. The incidence of such cases were higher in medulloblastomas (22%) and malignant gliomas (19%).

Spatial heterogeneity is indicated by the pattern of histograms obtained from different areas of the same tumour tissue, as shown for example in Fig 2. In one case (Fig. 2a), the relative proportions of the three cell populations having DNA indices 1.22 and 1.56 are quantitatively different in the three areas. Such regional variations were more frequently observed in gliomas as compared to other tumours. Another typical case is illustrated in Fig 2b. Here multiple DNA indices are not distinguishable but the relative proportions of cells in different phases of the cell cycle (G_1, S and G_2 + M) are different in various parts of

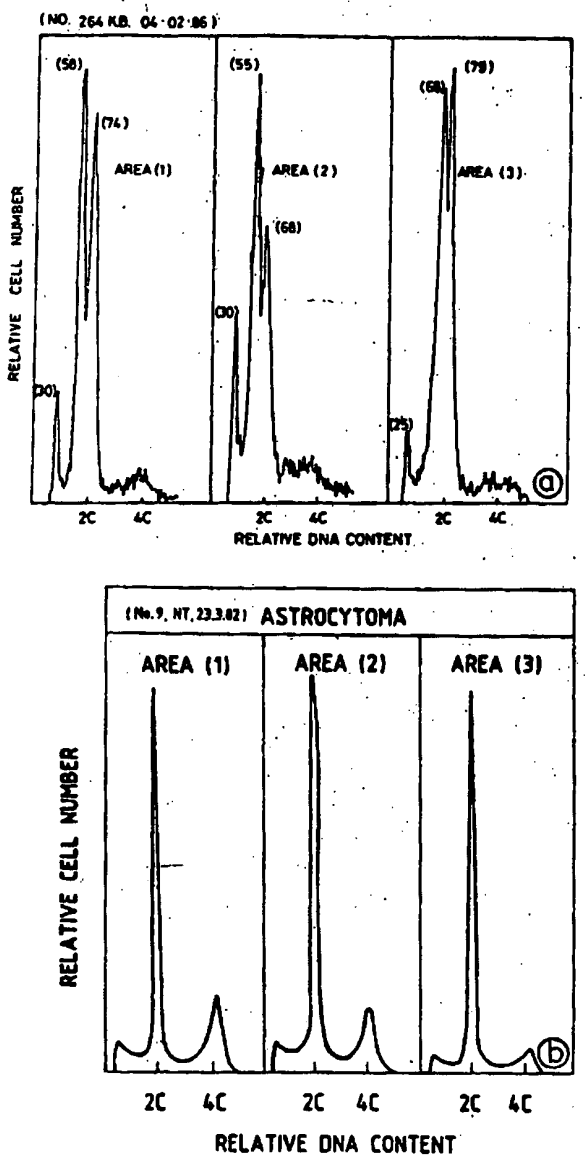

Fig. 2. DNA histograms of cells derived from different areas of a tumour showing spatial heterogeneity in (a) relative clonal composition (meningioma) and (b) proliferative status (astrocytoma IV).

the tumour. To obtain a quantitative estimate of heterogeneity among tumours, variance σ^2 (where σ is the standard deviation) of the mean value of PF was calculated. The values of σ^2 obtained for different types of tumours (Fig. 3) showed that spatial heterogeneity was higher in gliomas than in meningiomas and schwannomas.

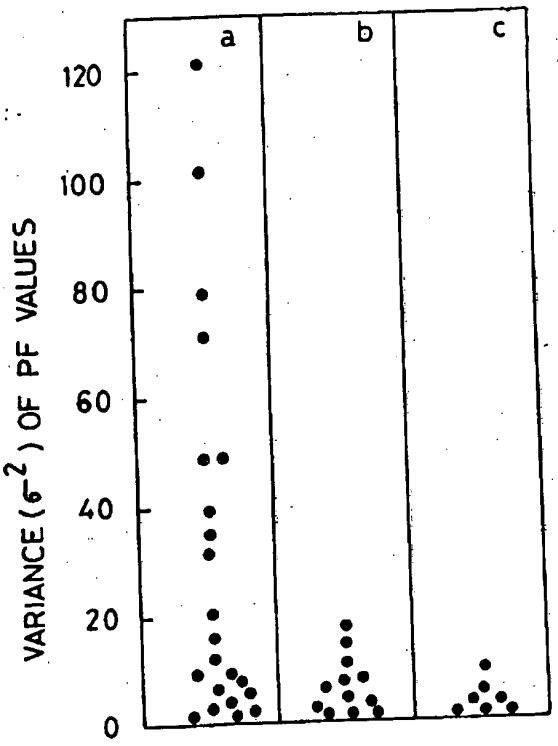

Fig. 3. Distribution of variance σ^2 observed in the % PF values of (a) glioma, (b) meningioma and (c) schwannoma. PF, proliferating fraction.

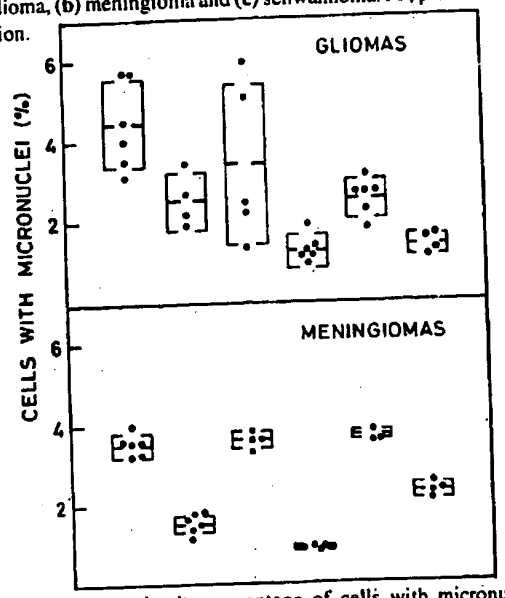

Fig. 4. Variations in the percentage of cells with micronuclei observed in different explants of the same tumour in organ culture. Two independent observers counted 1000 - 2000 cells from each explant.

Examples of M-fraction values observed in organ cultures of explants from different regions of the tumour tissue (glioma and meningioma) also indicated a higher degree of spatial heterogeneity in gliomas (Fig. 4). The values of variance (σ^2) in case of gliomas was generally higher by an order of magnitude (range 0.04 to 3.25) with an average value of 0.58 (n = 12) as compared to meningiomas (range 0.004 to 0.2) where the average value was 0.05 (n = 10).

Frequency distributions of DNA index (Fig. 5) among tumours showed that variations in the mean DNA contents of gliomas are larger than that of meningiomas and schwannomas (Table I). Further the distribution of DNA indices in higher grade (III/IV) gliomas showed a large range (0.9 to 3.6) than in lower grade I/II gliomas (1.6 to 2.6). The proliferative status as revealed by the values of S-fraction and proliferating fraction (PF) showed considerable amount of variation in the four types of primary brain tumours (Fig. 6 & Table II). The values of S-fraction and PF for gliomas which had a wider range as compared to meningiomas and schwannomas, also showed marked differences between grade III/IV (11 - 49%) and grade I/II (12 - 26%) tumours.

The mean values of M-fraction (MF%) analyzed in cells obtained from fresh tumour biopsy tissues

Table II. Inter-tumour variations in proliferative status of human brain tumours
(Data are mean±SD; range values in parentheses)

Tumour type	% SF	% PF
Glioma (n = 76)	11.0 ± 5.5 (1.2 - 27.4)	24 ± 8.2 (8.0 - 49.0)
Meningioma (n = 37)	8.5 ± 4.5 (2.3 - 20.3)	20.7 ± 7.5 (6.7 - 36.4)
Schwannoma (n = 24)	7.6 ± 2.9 (3.5 - 12.4)	18.5 ± 5.1 (8.0 - 30.7)
Medulloblastoma (n = 16)	8.0 ± 3.2 (3.7 - 13.6)	21.4 ± 6.8 (8.0 - 29.5)
Craniopharygioma (n = 7)	10.9 ± 4.3 (2.1 - 12.0)	23.1 ± 6.5 (13.7 - 26.2)
Others* (n = 28)	8.4 ± 3.4 (2.2 - 12.0)	16.9 ± 4.0 (8.7 - 21.2)

* Ependymoma, neurofibroma, haemangioblastoma tuberculoma *etc.* SF, % of cells in S-phase. PF, sum total of cells in S and G_2 + M phases

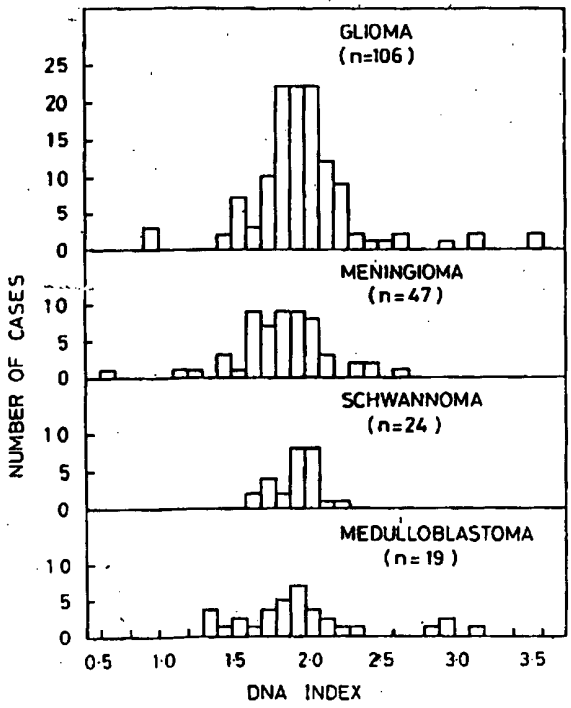

Fig. 5. Frequency distribution of DNA indices observed in four primary human brain tumours.

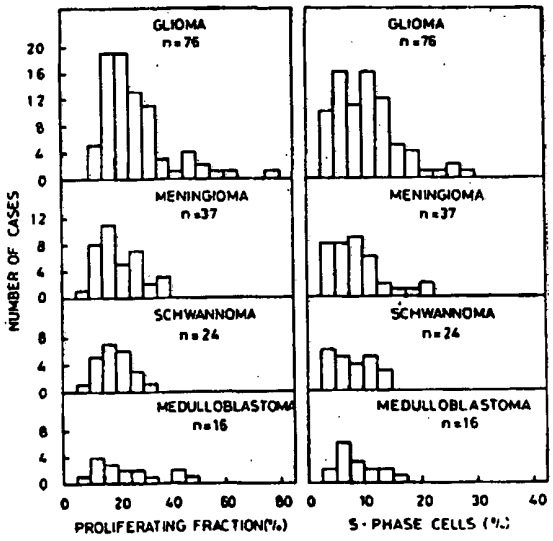

Fig. 6. Frequency distribution of proliferating fraction (%PF) and S-phase cells in four primary human brain tumours.

varied between 1.10 and 3.10 per cent in gliomas and between 0.46 and 3.80 per cent in case of meningiomas. The corresponding values at the end of 72 h in culture were 1.37 - 7.07 per cent in gliomas and 0.75 - 5.60 per cent in meningiomas.

Discussion

Lack of progress in the treatment of human brain tumours has prompted the investigation of heterogeneity of biological behaviour in these tumours[25,26] While, the relationship between many of the biological properties and tumour response to treatment has not always been unequivocal, a closer association between cytogenetic/cytokinetic parameters and response to treatment is seen in some patients[5,12,13]. Flow cytometry has revealed widely varying degrees of heteroploidy and proliferative activity in these tumours[16-19]. Results of the present studies confirm the heteroploid nature of malignant gliomas and also demonstrate, a higher degree of spatial heterogeneity in their cytokinetic organization. The incidence of aneuploid tumours and tumours with heteroploidy, reported here is likely to be an underestimate, as distinguishing tetraploid stem cells from G_2 + M fraction of the diploid clone is not always possible with DNA analysis alone[27].

The multiple peaks in the DNA histograms could arise on account of either subpopulation of cells with varying DNA contents or due to changes in the degree of chromatin condensation between cell populations with the same amount of DNA. The latter is more pertinent if the fluorochromes used have a structure dependent binding like for example the intercalating dyes ethidium bromide, propidium iodide *etc*. The multiploidy observed in this study is less likely to be due to such reasons since DAPI (an AT specific dye), is relatively insensitive to the degree of chromatin condensation[20]. It may also be mentioned that, use of internal standard (CE) and better measurement conditions (CV value of 0.9 to 1.2% for human PBL) has allowed us to resolve small differences in the cellular DNA content, unlike some of the earlier studies[12,13,16,17].

Regional variations in the cytokinetic parameters (spatial heterogeneity) as observed by us could arise in solid tumours on account of gradients in the nutritional status across the tumour due to vascular

deformities[28]. In case of gliomas, however, the infiltrating nature of the tumour cells could also contribute to this phenomenon. Variations in the values of M-fraction between different explants of the same tumour in culture observed in case of gliomas could be partly due to variations in the growth fraction in culture, as has been observed earlier[29]. Part of the variation could also be due to differences in the distribution of cell populations with varying degrees of chromosomal instability and proliferation across the tumour. Consequently, the responses of cells at different sites within the tumour are also likely to vary. This points towards the need for multiple sampling from defined areas of the tumour, while carrying out *in vitro* sensitivity tests.

The explanation for a wide distribution of DNA index values in brain tumours as observed here and reported earlier[16-19] is still obscure. Variations in DNA index could possibly arise on account of variations in the chromosomal instability associated with the malignant transformation. Therefore, identification of multiple clones and precise estimation of deviation from diploid DNA content would complement clinicopathological findings in aiding diagnosis and could also be helpful in designing the most effective treatment. Further, since the clones can be sorted based on their DNA content, their response to treatment could be evaluated *in vitro*[6] before administering the treatment.

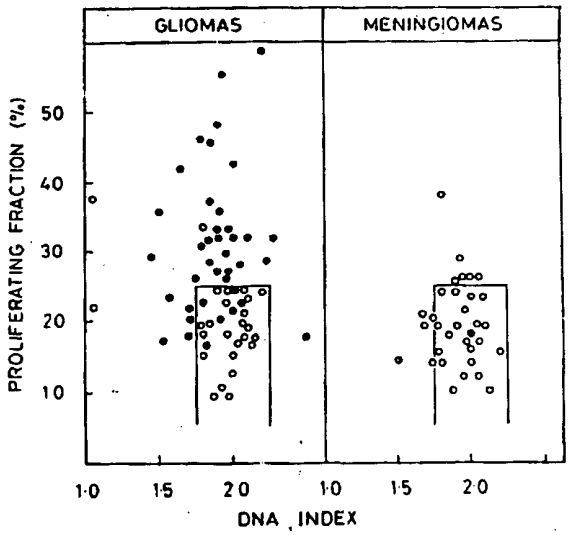

Fig. 7. Bivariate analysis of gliomas with DNA index and %PF values. O : low grade tumours; • : high grade tumours.

Analysis of the intertumoral heterogeneity, shows that, distinguishing low (I/II) and high (III/IV) grade gliomas (in comparison with the histopathological diagnosis) based only on one parameter *i.e.*, DNA index or % PF is often very difficult. A bivariate analysis taking both DNA index and per cent PF value (Fig. 7) could be more useful in this regard. More than 97 per cent of the low grade gliomas showed values within the area circumscribed by DNA index values in the range 1.8 - 2.2 and PF < 25 per cent, designated as less aggressive flow cytometrically. On the other hand nearly 20 per cent of the high grade gliomas were also found in this area. However, combining the information on ploidy status (especially the multiploid nature) with the bivariate analysis, facilitated the process of distinguishing low and high grade tumours in more than 90 per cent instances.

In conclusion, it may be stated that analysis of DNA content, proliferative characteristics and micronuclei expression taking into account the clonal diversity and spatial heterogeneity would provide a greater insight into the malignant behaviour of human brain tumours. This would not only supplement histopathological diagnosis but could also be useful in prognosis and individualization of therapy.

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Reprint requests : Dr Viney Jain, Director, Institute of Neuclear Medicine and Allied Sciences, Lucknow Road, Delhi 110054

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RESEARCH ARTICLES

Cytogenetic Analysis of Pancreatic Carcinomas: Intratumor Heterogeneity and Nonrandom Pattern of Chromosome Aberrations

Ludmila Gorunova,[1]* Mattias Höglund,[1] Åke Andrén-Sandberg,[2] Sigmund Dawiskiba,[3] Yuesheng Jin,[1] Felix Mitelman,[1] and Bertil Johansson[1]

[1]Department of Clinical Genetics, University Hospital, Lund, Sweden
[2]Department of Surgery, University Hospital, Lund, Sweden
[3]Department of Pathology and Cytology, University Hospital, Lund, Sweden

Twenty-nine nonendocrine pancreatic carcinomas (20 primary tumors and nine metastases) were studied by chromosome banding after short-term culture. Acquired clonal aberrations were found in 25 tumors and a detailed analysis of these revealed extensive cytogenetic intratumor heterogeneity. Apart from six carcinomas with one clone only, 19 tumors displayed from two to 58 clones, bringing the total number of clones to 230. Karyotypically related clones, signifying evolutionary variation, were found in 16 tumors, whereas unrelated clones were present in nine, the latter finding probably reflecting a distinct pathogenetic mechanism. The cytogenetic profile of pancreatic carcinoma was characterized by multiple numerical and structural changes. In total, more than 500 abnormal chromosomes, including rings, markers, homogeneously stained regions, and double minutes, altogether displaying 608 breakpoints, were detected. This complexity and heterogeneity notwithstanding, a nonrandom karyotypic pattern can be discerned in pancreatic cancer. Chromosomes 1, 3, 6, 7, 8, 11, 12, 17, and 19 and bands 1q12, 1q21, 3q11, 6p21, 6q21, 7q11, 7q22, 7q32, 11q13, 13cen, 14cen, 17q11, 17q21, and 19q13 were most frequently involved in structural rearrangements. A total of 19 recurrent unbalanced structural changes were identified, 11 of which were not reported previously: del(1)(q11), del(3)(p11), i(3)(q10), del(4)(q25), del(11)(p13), dup(11)(q13q23), i(12)(p10), der(13;15)(q10;q10), del(18)(q12), del(18)(q21), and i(19)(q10). The main karyotypic imbalances were entire-copy losses of chromosomes 18, Y, and 21, gains of chromosomes 7, 2, and 20, partial or whole-arm losses of 1p, 3p, 6q, 8p, 9p, 15q, 17p, 18q, 19p, and 20p, and partial or whole-arm gains of 1q, 3q, 5p, 6p, 7q, 8q, 11q, 12p, 17q, 19q, and 20q. In general, the karyotypic pattern of pancreatic carcinoma fits the multistep carcinogenesis concept. The observed cytogenetic heterogeneity appears to reflect a multitude of interchangeable but oncogenetically equivalent events, and the nonrandomness of the chromosomal alterations underscores the preferential pathways involved in tumor initiation and progression. *Genes Chromosomes Cancer 23:81–99, 1998.* © 1998 Wiley-Liss, Inc.

INTRODUCTION

Pancreatic carcinoma is one of the leading causes of cancer-related death in virtually all industrialized countries and is characterized by aggressive tumor growth and a high metastatic propensity (Warshaw and Fernández-del Castillo, 1992). The biology and pathogenesis of this cancer are poorly understood. However, genetic research during the last few years, by both chromosome banding and molecular techniques, has provided some insights into the key events underlying pancreatic carcinogenesis. It has become clear that, although this cancer is accompanied by numerous acquired genetic alterations, certain changes seem to occur nonrandomly. On the cytogenetic level, the most consistent abnormalities include gain of chromosomes 7 and 20 and loss of 18, Y, 13, 12, 17, and 6, as well as overrepresentation of 1q, 8q, and 17q and underrepresentation of 1p, 6q, 8p, and 17p (Bardi et al., 1993; Griffin et al., 1994, 1995).

Loss of heterozygosity (LOH) analyses in pancreatic carcinoma, corroborating and extending the karyotypic results, have demonstrated frequent allelic imbalances at 1p, 3p, 6p, 6q, 8p, 9p, 10q, 12q, 13q, 17p, 18q, 21q, and 22q (Seymour et al., 1994; Hahn et al., 1995; Kimura et al., 1996), indicating regions likely to harbor tumor suppressor genes (TSGs). Indeed, some TSGs, for example, *CDKN2A* at 9p21 (Caldas et al., 1994; Bartsch et al., 1995), *BRCA2* at 13q12 (Goggins et al., 1996), *TP53* at 17p13 (Ruggeri et al., 1992; Scarpa et al., 1993), and *SMAD4* (formerly *DPC4*) at 18q21 (Hahn et al., 1996), have been implicated in pancreatic tumorigenesis. The other type of cancer-related genes, the dominantly acting oncogenes, has also been associated with this malignancy, in particular *KRAS2*,

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*Correspondence to: Dr. Ludmila Gorunova, Department of Clinical Genetics, University Hospital, S-221 85 Lund, Sweden.

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TABLE I. Clinical and Histopathologic Data on the Pancreatic Carcinomas

Case No./ Lab No.	Sex/ Age	Morphology[a]	Grade[b]	Site[c]	Case No./ Lab No.	Sex/ Age	Morphology[a]	Grade[b]	Site[c]
1/2351-92	M/68	D	WD	Caput	15/2847-92	M/70	C	MD-PD	Caput
2/3053-92	F/54	D	MD	Corpus	16/1173-93	M/71	C	PD	Cauda
3/3304-92	F/58	D	PD	Caput	17/1707-93	F/76	C	PD	NOS[d]
1853-93	59			Caput[d]	18/2238-93	M/72	C	PD	NOS[d]
4/3324-92	M/63	D	PD	Corpus	19/2378-93	M/50	C	PD	PV[d]
5/951-93	F/60	D	WD	Caput	20/2517-93	F/69	C	PD	NOS[d]
6/1727-93	F/78	D	PD	Corpus	21/2605-93	M/66	C	PD	Corpus[d]
7/2211-93	F/58	D	WD-PD	Corpus[d]	22/630-94	M/50	C	PD	Caput[d]
8/2696-93	F/66	D	MD	Caput-Corpus	23/2913-92	M/64	SCC	PD	Caput-Corpus
9/3378-93	F/76	D	PD	Caput	24/979-93	F/45	SCC	PD	NOS[d]
10/779-94	M/61	D	MD-PD	Corpus-Cauda	25/2620-93	M/62	CA	WD	Corpus
11/785-94	F/54	D	MD	Corpus	26/1330-94	F/53	IPMC	WD	Caput[e]
12/1174-94	F/47	D	WD-MD	Caput	27/1258-94	F/54	MCA	WD	Caput-Corpus
13/1298-94	M/49	D	WD-PD	Caput	28/129-94	M/68	Inc	—	Caput
14/2033-92	F/74	C	PD	PV					

[a]D, ductal cancer; C, carcinoma; SCC, small cell carcinoma (negative for neuroendocrine granules); CA, cystadenocarcinoma; IPMC, intraductal papillary-mucinous carcinoma; MCA, mucinous cystadenocarcinoma; Inc, inconclusive (final diagnosis of pancreatic cancer was based on perioperative findings).
[b]WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.
[c]PV, papilla Vateri; NOS, pancreas, not otherwise specified.
[d]Sample obtained from abdominal metastatic lesion.
[e]Multicentric origin from pancreas, papilla Vateri, and choledochus.

which is mutated in most cases (Almoguera et al., 1988; Hruban et al., 1993).

Recently, comparative genomic hybridization (CGH) has complemented conventional cytogenetics in the characterization of chromosomal imbalances in pancreatic carcinoma. Although different CGH analyses (Solinas-Toldo et al., 1996; Fukushige et al., 1997; Mahlamäki et al., 1997) have yielded partly discordant results, the most common alterations have been gain of 20q, 8q, 11q, and 17q and loss of 9p, 6q, and 18q.

In view of the complexity of the genetic profile of pancreatic carcinoma and the somewhat conflicting data about the malignancy-relevant alterations, further studies are warranted. In this context, cytogenetic techniques remain instrumental for genome-wide screening of aberrations, providing, at the same time, the possibility of monitoring cell-to-cell variability. Here, we report data on 29 consecutive tumors (three of which were previously published) as part of an ongoing genetic study of pancreatic carcinoma, specifically addressing the intratumor karyotypic heterogeneity which, to date, is poorly documented in this malignancy.

MATERIALS AND METHODS

Clinical and Histopathologic Data

Forty-three tumor specimens of nonendocrine pancreatic cancer were obtained at surgery between August 1992 and May 1994. None of the patients had received prior genotoxic therapy. Fourteen samples did not yield any cytogenetic results because of infection or failure to grow in short-term culture. For the remaining 29 tumors from 28 patients, clinical and histopathologic data, including sex, age, tumor morphology, grade, and site, are presented in Table 1. Nine of the tumor specimens were taken from abdominal metastatic lesions. When carcinomas were unresectable, i.e., when pancreatectomy could not be performed, the final diagnosis was based on pre- and perioperative examinations.

Cytogenetic Methods

For the cytogenetic analysis, a piece of tumor tissue was taken close to that used for histologic examination. In cases 5 and 15, only needle (Tru-Cut) biopsies were available. The specimens were washed in RPMI 1640, minced with scalpels, and enzymatically disaggregated in 1,400 U/ml collagenase II for 2–3 hours; for needle biopsy specimens and very small samples, the disaggregation time was reduced to 30–45 min. The resulting suspension was washed twice by centrifugation in RPMI 1640. The final pellet was resuspended in culture medium and plated in 25 cm^2 plastic flasks coated with Vitrogen 100. The culture medium was DME/F12 (1:1) supplemented with 10 μg/ml ascorbic acid, 0.5 μg/ml cholera toxin, 10 nM dibutyryl cAMP, 20 ng/ml epidermal growth factor, 20 μg/ml

fetuin, 100 ng/ml fibronectin, 2 mM glutamine, 0.5 µg/ml hydrocortisone, 3 µg/ml insulin, 0.1 mM phosphoethanolamine, 2.6 ng/ml sodium selenite, trace element mix (1:100), 25 µg/ml transferrin, 10 nM triiodothyronine, and 10% fetal bovine serum. Penicillin (100 IU/ml) and streptomycin (100 µg/ml) were added to all media used.

After 1–3 weeks, a period depending on the amount of the initial cell inoculate, cultures were exposed to 0.015 µg/ml of Colcemid for 3–14 hours and harvested 1–3 times each. Cells were detached from the surface by trypsin/EDTA and, after immersion in 0.06 M KCl for 30 min followed by repeated fixations, were dropped onto wet slides. The air-dried slides were kept at 60°C overnight and then incubated in 2 × SSC at 60°C for 4 hours. The chromosomes were G-banded with Wright's stain. In the cytogenetic analysis, the clonality criteria and karyotypic description followed the ISCN (1995). To increase the probability of finding even small clones, we analyzed as many (11–1,175) metaphases as possible per tumor.

For assessment of the identified chromosomal changes in a more comprehensive way, the data are also presented as breakpoint and karyotypic imbalance maps. In order to record the basic and yet representative spectrum of aberrations and to avoid cytogenetic "noise" caused by excessive chromosome numbers, we included only 2n–4n clones for each tumor in the maps. If more than one copy of the same aberration was found in the same clone or if the same aberration was found in more than one related clone, the breakpoints involved were plotted only once. If an aberrant chromosome was further rearranged in the same or related clones, only additional breakpoints were plotted. If a tumor had more than one clone, related or unrelated, chromosomal imbalances were recorded in each of them separately and in relation to the nearest euploid level. When the same chromosome was involved in both numerical and structural aberrations, the total net imbalance was recorded. The same imbalance found in more than one related clone was recorded only once. If additional gains or losses of the same chromosome or chromosomal segment were found in related clones, only the largest imbalance was recorded.

RESULTS

Clonal chromosome changes were detected in 25 (86%) of the 29 tumors; the remaining four were karyotypically normal. Of the 25 abnormal carcinomas (Table 2, Figs. 1 and 2), six had one clone, whereas 19 displayed from two to 58 aberrant clones, yielding a total of 230 clones. In addition, normal metaphases were seen in all cases. Karyotypically related clones (subclones) were present in 16 tumors and unrelated clones in nine. Six carcinomas were near-diploid, three were near-triploid, and the remaining 16 tumors had clones with ploidy levels spanning 2n–17n. Among the 230 aberrant clones, 136 (60%) had complex karyotypes (defined here as more than three structural chromosomal changes), and 94 exhibited simple deviations. Only karyotypically simple clones were found in six carcinomas, whereas such clones were present together with complex clones in 13 cases.

All chromosomes were affected by both numerical and structural changes. Structural alterations were multiple and diverse, giving rise to 511 different abnormal chromosomes, including 19 rings and 34 markers. Apart from the two latter types of abnormality, more than 70% of the aberrant chromosomes were involved in unbalanced rearrangements. The proportion of such chromosomes in karyotypically complex clones was greater than 90%, whereas in clones with simple changes it was less than 15%. Double minute chromosomes and homogeneously stained regions (hsr) were observed in four and three cases, respectively. Altogether, 19 recurrent structural aberrations, all of them unbalanced, were found: del(3)(p11) and i(5)(p10) in five tumors; i(1)(q10), del(1)(q12), i(3)(q10), and del(9)(p13) in three tumors; and del(1)(q11), del(1)(q21), del(4)(q21), del(4)(q25), del(6)(q15), i(8)(q10), del(11)(p13), dup(11)(q13q23), i(12)(p10), der(13;15)(q10;q10), del(18)(q12), del(18)(q21), and i(19)(q10) in two tumors (Fig. 3).

A total of 608 chromosomal breakpoints were identified in 23 carcinomas (Fig. 4), with up to 66 different breakpoints per clone (case 17) and up to 129 per tumor (case 16). Chromosome 1 displayed the highest number of structural changes (58 breakpoints), followed by chromosomes 6, 7, 3, 17, 8, 11, 12, and 19 (52–28 breakpoints). The bands preferentially involved (≥7 breakpoints) were 1q12, 1q21, 3q11, 6p21, 6q21, 7q11, 7q22, 7q32, 11q13, 13cen, 14cen, 17q11, 17q21, and 19q13.

The most frequent entire copy number imbalances were, in decreasing order, losses of chromosomes 18, Y, 21, X, and 9 and gains of chromosomes 7, 2, 20, 11, 12, and 16. The most common partial or whole-arm losses affected 1p, 3p, 6q, 8p, 9p, 15q, 17p, 18q, 19p, and 20p, as well as the short arms of the acrocentric chromosomes. The most frequent partial or whole-arm gains involved 1q, 3q, 5p, 6p, 7q, 8q, 11q, 12p, 17q, 19q, and 20q (Figs. 3 and 5).

TABLE 2. Cytogenetic Findings in the Pancreatic Carcinomas

Case No./ Lab No.	Karyotype[a]	No. of abnormal clones unrelated: related (subclones) [No. of cells analyzed]
1/2351-92[b]	45,X,−Y[12]/46,XY[40]/nonclonal[2]	1 [54]
3/3304-92	47,XX,+7[31]/46,XX[116]/nonclonal[4]	1 [151]
/1853-93	68–69,XXX,der(1)t(1;8)(q21;q11),der(1)t(1;8)add(1)(p13),+2,−5,−6,−8,−9,del(10)(p13),+11,+12,der(12)t(1;12)(q21;q21),der(12)t(1;12)add(12)(p13),+14,der(15)t(15;19)(p13;q12)add(19)(q13),−17,−18,−18,add(19)(q13),+20,−21,+22,+r,+1–2mar[42]/69–71,idem,+add(16)(p13)[4]/136–138,idem×2[13]/200–300,idem×3–4,dmin[5]	4 −:4 [64]
4/3324-92	42,X,−Y,t(3;5)(q23;q13),−6,−13,der(17)t(6;17)(p11;p11),−18,der(19)t(X;19)(q21;p13)[3]/43,idem,+**del(18)(q12)**[32]/43,idem,+r1[7]/43,idem,r(11),+r1[2]/43,idem,−9,+del(18)(q12),+r1[4]/43,idem,+del(18)(q12),−21,+r1[5]/43,idem,dic(3;18)(q29;q12),+r1[2]/43,idem,+dic(18;!;20)(q12;?;q13),−20,+r1[2]/43,idem,+dic(18;?;21)(q12;?;p13),−21,+r1[21]/44,idem,+add(18)(q12),+r1[3]/44,idem,+del (18)(q11),+r1[4]/44,idem,+del(18)(q12),+r1[228]/44, idem,+der(18)t(3;18)(p11;q12),+r1[10]/44,Idem,**del(3)(p11)**,+del(18)(q12),+r1[2]/44,idem,del(3)(p14),+del(18)(q12),+r1[2]/44,idem,del(10)(p11),+del(18)(q12),+r1[2]/44,idem,del(11)(q14),+del(18)(q12),+r1[3]/44,idem,+r1,+r2[14]/51–57,idem,+1,+2,+3,+4,+der(5)t(3;5),+6,+8,+8,+9,+10,+12,+13,+14,+15,+16,+der(17)t(6;17),+18,del(18)(q12)×2,+20,+22,+r1[5]/69–78,XX,−Y,+1,+3,t(3;5)×2,+5,−6,+7,+10,+12,−13,+14,+15,+16,+17,der(17)t(6;17)×2,+18,del(18)(q12)×2,der(19)t(X;19)×2,+20,+21,+22,+r1×2[11]/88,Idem×2,+r1×2,+r2×2[3]/81–98,idem×2,+8,+dic(18;?;21)×2,+r1×2[5]/82–85,idem×2,+del(18)(q11)×2,+r1×2[3]/81–100,idem×2,+10,+del(18)(q12)×2,+r1×2[57]/45, X,−Y[15]/46, XY,**del(1)(q12)**[2]/46,XY,t(7;17)(q11;q11)[2]/46,XY[183]/nonclonal [24]	27 3:25+1+1 [656]
6/1727-93	78–80,XXX,+X,+X,**i(1)(q10)**,+2,+3,**del(3)(p11)**×2,i(6)(p10),+7,der(7)t(7;11)(q31;q13)dup(11)(q13q13)×2,der(8)t(7;8)(q22;q12),+der(8)t(8;8)(p11;q21)×2,**del(9)(p13)**,+12,−13,+14,−15,+16,der(17)i(17)(q10)add(17)(q25),−18,+19,**i(19)(q10)**×2,+20,−21,+1–2mar[17]/78–80,idem,+**i(12)(p10)**[11]/65–73,XX,del(X)(q24),i(1)(q10),−3,**del(4)(q25)**×2,i(4)(q10),−6,der(7)t(7;11),der(8)t(7;8),+der(8)t(8;8)×3,+der(8)t(8;8)t(8;15)(q24;q21),−9,del(9),+10,del(11)(q21),i(12)(p10),−13,i(13)(q10),−15,i(15)(q10),+16,?hsr(17)(q21),−18,add(19)(q13),der(19)t(11;19)(q21;q13),i(19)(q10),+20,+3–4mar[11]/130–140,XXXX,del(X)×2,i(1)(q10)×2,−3,−3,del(4)×4,i(4)(q10)×2,−6,−6,der(7)t(7;11)×2,der(8)t(7;8)×2,+der(8)t(8;8)×3,+der(8)t(8;8)t(8;15)×2,−9,−9,del(9)×2,del(11)×2,−13,−13,−15,−15,i(15)(q10)×2,+16,+16,+16,−17,?hsr(17)×2,−18,−18,−19,−19,i(19)(q10)×2,+3–4mar,dmin[9]/156–160,idem×2[21]/156–160,idem×2,+i(12)(p10)×2[17]/46,XX[25]	6 −:6 [111]
7/2211-93	70–74,XX,−X,add(1)(p34),add(1)(q25),+2,**i(3)(q10)**,add(5)(q11)×2,der(6)t(6;11)(q21;q21),del(7)(q31q34)×2,+8,−9,del(9)(q32),+add(11)(q21),+12,+13,del(15)(q22q24),+16,+17,der(17)t(5;17)(q13;p13)×2,add(18)(q12),−19,+20,−21[36]/72–73,idem,+der(7)t(7;12)(p13;p11)[7]/68–73,idem,add(12)(q24)[5]/ 89–95,XXX,−X,add(1)(p34),add(1)(q25),+2,i(3)(q10),add(5)×2,der(6)t(6;11)×2,del(7)×2,+8,−9,del(9),+11,add(11)×2,+13,+13,del(15),+16,+17,+17,der(17)t(5;17)×3,+18,add(18),−19,−21,+22[3]/140–148,idem×2,+der(7)t(7;12)×2,add(12)×2[21]/210–280,idem×3–4,+der(7)t(7;12)×3–4[10]/46,XX[28]/nonclonal [1]	6 −:6 [111]
9/3378-93	40–41,X,del(X)(p11),−1,**del(3)(p11)**,−6,−9,del(10)(q22),−12,der(15)t(6;15)(p11;p11),add(16)(p13),der(17)inv(17)(p11q21)ins(17;7)(p11;q11q22),−18,der(19)t(1;19)(q12;q13),add(20)(p13),−21,add(21)(p11)[7]/68–80,X,+X,del(X)×2,−1,+2,+3,del(3)×2,+4,t(4;10)(q25;q26)×2,−5,−6,+7,+del(7)(q11),der(8;14)(q10;q10),−9,+11,−12,+13,+14,+15,der(15)t(6;15)×2,+16,add(16)×2,+17,+17,der(17)inv(17)ins(17;7),der(17)inv(17)ins(17;7)del(7)(q11)×2,−18,+19,der(19)t(1;19)×2,+20,add(20)×2,−21,add(21)×2,+22[cp9]/120–150,XXX,del(X)×3,−1,−1,+2,+2,+3,+3,del(3)×4,+4,t(4;10),der(4)t(4;10)×3,+5,−6,−6,+del(7)×2,+8,+8,+der(8;14)×2,−9,−9,del(10)(q22)×2,+11,−12,−12,+13,+13,der(15)t(6;15)×3,add(16)×3,+17,+17,der(17)inv(17)ins(17;7)×4,−18,−18,+19,+19,der(19)×2,+20,+20,add(20)×4,−21,−21,−21,add(21)×3[cp6]/200–300,idem×5–7,t(4;10)×4,der(4)t(4;4)(q31;q25)×3,del(7)×4,der(8;14)×4,−der(17)inv(17)ins(17;7),+der(17)inv(17)ins(17;7)del(7)×6,+2–4r[cp4]/47,XX,+7[33]/47,XX,+20[3]/46,XX[70]/nonclonal[14]	6 −:6 [146]

TABLE 2. Cytogenetic Findings in the Pancreatic Carcinomas (continued)

Case No./ Lab No.	Karyotype[a]	No. of abnormal clones unrelated: related (subclones) [No. of cells analyzed]
10/779-94	78–80,X,−X,−Y,+**i(1)(q10)**,+2,+3,der(3;13)(q10;q10)×2,+4,+**i(5)(p10)**×2,+7,+8,−10,+**del(11)(p13)**,+der(11;19)(p10;q10),+12, der(13;21)(q10;q10),+14,add(14)(p11)×2,−15,+16,+17,−18,+**i(19)(q10)**,+20,+20,−22,add(22)(p11)×2,+r,+3mar[cp6]/46,XY[7]/ nonclonal[2]	1 [15]
12/1174-94	72–81,XX,−X,+1,dic(1;?)(p11;?)×2,+2,inv(2)(p11q31)×2,+3,del(3)(p13p23)×2,+5,**i(5)(p10)**×2,del(6)(q25),+**del(6)(q15)**×2,+7,+8, del(8)(p21)×2,add(9)(q32)×2,**del(9)(p13)**,+del(9),+10,add(10)(q22)×2,+11,−12,der(13;14)(q10;q10)×2,**der(13;15)(q10;q10)**, del(14)(q24),+16,−18,−18,−18,+19,+20,−21,der(21)t(1;21)(p22;p13),+22,+4mar[cp5]/46,XX[62]/nonclonal[7]	1 [74]
13/1298-94[c]	38,X,−Y,del(2)(q33q35),der(6)r(6)(p25q21)ins(6;?)(q13;?),add(8)(p11),der(9)t(9;17)(p11;q11),der(10)t(10;17)(p11;q11),−13,der(14; 22)(q10;q10),−15,−17,−18,der(19;21)(q10;q10)del(19)(q13),−21[60]/37,idem,−der(6)r(6)ins(6;?)[8]/37–39,idem,**del(1)(q11)**[2]/38, idem,add(7)(q32)[2]/37–38,idem,add(7)(q32)[3]/35–38,idem,add(7)(q32)[4]/37–38,idem,add(7)(q32)[4]/38,idem,del(7)(q32)[2]/38,idem, **dup(11)(q13q23)**[2]/38–39,idem,der(X)t(X;7)(p22;p11),der(6)t(6;19)(p21;q13),−7,der(7)t(6;7)(p21;q36),−der(14;22),+r1,+mar1[4]/38–39,idem,der(7)(pter→q32::q11→q22::q11→q32::q32→q22::?::19q11→19qter)[290]/37–38,idem,−der(6)r(6)ins(6;?),der(7)(pter→q32::q11→q22::q11→q32::q32→q22::?::19q11→19qter)[16]/39–40,idem,**i(5)(p10)**,−der(10)t(10;17),+i(10)(q10),+der(16)t(5;16)(q22;q13), +add(17)(p11),−20[4]/38,idem,−der(10)t(10;17),+der(17;20)(q10;q10),+der(18)t(10;18)(q11;q11),−20[5]/38,idem,−der(10)t(10;17), −der(14;22),+der(14;22)add(22)(q13),+der(17;20),+der(18)t(10;18),−20[13]/40,idem,−der(6)r(6)ins(6;?),+ins(6;?)(q13;?),−der(10) t(10;17),+i(10)(q10),+12,+add(17)(p13),−20,der(22)t(20;22)(q11;p11),+mar2[27]/40,idem,−der(6)r(6)ins(6;?),+ins(6;?),−der(10)t(10; 17),+i(10)(q10),+12,+der(17)r(17;?)(p13q25;?),−20,der(22)t(20;22),+mar2[28]/39–40,idem,inv(4)(p11q27),−der(6)r(6)ins(6;?),+ins(6; ?),−der(10)t(10;17),+i(10)(q10),+12,+der(17)r(17;?),−20,der(22)t(20;22),+mar2[3]/39,idem,der(3)r(3;?),−der(6)r(6)ins(6;?),+ins(6;?), −der(10)t(10;17),+i(10)(q10),+der(17;20),−20,+mar2[4]/76–77,idem×2[cp12]/73–75,idem×2,der(X)t(X;7)×2,der(6)t(6;19)×2,−7, −7,der(7)t(6;7)×2,der(14;22)×2,+2r1[3]/73−76,idem×2,der(7)(pter→q32::q11→q22::q11→q32::q32→q22::?::19q11→19qter)×2 [cp114]/73–74,idem×2,der(7)(pter→q32::q11→q22::q11→q32::q32→q22::?::19q11→19qter)×2,−der(6)r(6)ins(6;?)[cp98]/78–80, idem×2,−der(6)r(6)ins(6;?)×2,+ins(6;?)×2,−der(10)t(10;17)×2,+i(10)(q10)×2,+12,+12,+add(17)(p13)×2,−20,−20,der(22)t(20; 22)×2,+mar2×2[10]/79–80,idem×2,−der(6)r(6)ins(6;?)×2,+ins(6;?)×2,−der(10)t(10;17)×2,+i(10)(q10)×2,+12,+12,+der(17)r(17; ?)×2,−20,−20,der(22)t(20;22)×2,+mar2×2[14]/66–74,idem×2,−1,**del(4)(q21)**,−der(6)r(6)ins(6;?),−der(10)t(10;17)×2,+i(10)(q10), −der(14;22),+add(17)(p11)×2[cp6]/72–74,idem×2,−1,del(4),−der(6)r(6)ins(6;?),−der(10)t(10;17)×2,+i(10)(q10),−der(14;22),+i(17)(q10)[cp3]/72– +i(14)(q10)[cp11]/73–74,idem×2,−1,del(4),−der(6)r(6)ins(6;?),−der(10)t(10;17)×2,+i(10)(q10),−der(14;22),+i(17)(q10)[cp3]/72– 73,idem×2,−1,−der(6)r(6)ins(6;?),−der(10)t(10;17)×2,+der(10)t(10;19)(p11;q11),−der(14;22)[cp65]/69–73,idem×2,−1,−der(6)r(6) ins(6;?),−der(10)t(10;17)×2,+der(10)t(10;19),−der(14;22),t(16;17)(q24;q21)[cp130]/72–73,idem×2,−1,−der(6)r(6)ins(6;?),−der(10) t(10;17)×2,+der(10)t(10;19),−der(14;22),+mar3,+mar4[cp9]/72–73,idem×2,−1,−der(6)r(6)ins(6;?),−der(10)t(10;17)×2,+der (10)t(10;19),−der(14;22)×2,+i(14)(q10)[2]/150–300,idem×4–8[cp10]/150–200,idem×4–5,del(4),−der(10)t(10;17)×4–5,+i(10)(q10) ×2–3,−der(14;22)×4–5,+i(14)(q10)×2–3[6]/160–400,idem×4–10,der(7)(pter→q32::q11→q22::q11→q32::q32→22::?::19q11→19qter) ×4–10[cp102]/158–300,idem×4–8,−der(6)r(6)ins(6;?)×4–8,+ins(6;?)×4–8,−der(10)t(10;17)×4–8,+i(10)(q10)×4–8,+12,+12,+12, +12,+12,+12,+add(17)(p13)×2–4,+der(17)r(17;?)×2–4,−20,−20,−20,−20,−20,−20,der(22)t(20;22)×4–8,+mar2×4–8[cp9]/150–400, idem×4–10,−der(10)t(10;17)×4–10,+der(10)t(10;19)×2–5,−der(14;22)×2–5,+t(16;17)×2–5,+mar3×2–5,+mar4×2–5[cp22]/45, X,−Y[7]/47,XY,+8[3]/46,XY[54]/nonclonal[4]	39 −:39 [1175]
14/2033-92	71–72,XXX,+X,del(1)(p32)×2,der(1)t(1;7)(p31;q11),+der(1)t(1;7),+der(1)t(1;3)(q21;p23)×2,+2,−3,**del(3)(p11)**×2,−4,−5,−6,+7, der(7)t(3;7)(q21;q11)×2,−11,−12,−14,+16,−17,−18,−18,+19,−22,+2mar,inc[8]/46,XX[3]	1 [11]

TABLE 2. Cytogenetic Findings in the Pancreatic Carcinomas (continued)

Case No./ Lab No.	Karyotype[a]	No. of abnormal clones unrelated: related (subclones) [No. of cells analyzed]
15/2847-92[d]	60–69,XX,−Y,+**i(1)(q10)**,+del(2)(p13),−3,−4,+6,del(6)(p23)×2,+7,+8,der(8)t(8;13)(p23;q14)×2,−9,der(9)t(9;11)(p13;q11)×2,−11, add(11)(q21),idic(11)(q14),idic(12)(p12),−13,−13,−13,idic(14)(p13),dic(15;15)(p11;p11)del(15)(q11q21)del(15)(q11q21),−17,der(17) t(13;17)(q12;p13)×2,−18,**del(18)(q21)**,+19,der(19)t(3;19)(q11;p13)×2,+20,+r,+mar[195]/64–68,idem,−X,+Y[5]/51–68,idem,**del(1) (q12)**[13]/66–68,idem,del(1)(q12),−del(6)(p23),+der(6)del(6)(p23)del(6)(q15)[2]/65–82,idem,del(1)(p11),−i(1)(q10)[3]/66–69,idem, +add(1)(p13),del(1)(p11),−i(1)(q10)[3]/63–79,idem,−del(6)(p23),der(1)t(1;6)(q21;p11)del(6)(p23)[3]/59–66,idem,−1,−del(6)(p23), +der(6)del(6)(p23)del(6)(q13q21)[3]/60–69,idem,2–4dmin[3]/120–138,idem×2[43]/120–138,idem×2,dmin[7]/45,X,−Y[14]/46,XY, t(2;8)(q31;p23)[11]/46,XY[40]	13 / 2:12+1 [345]
16/1173-93[e]	46,XY,der(1)del(1)(q11q21)del(1)(q25q32),t(8;14)(p21;q32),ins(9;?)(q12;?)[2]/46,idem,**del(9)(p13),dup(11)(q13q23)**[7]/46,XY,der(1),der(3) t(3;11)(q21;q13),**del(4)(q21)**,der(7)t(3;7)(q21;q36),der(8)t(8;14)t(4;8)(q25;q22),ins(9;?),der(14)t(8;14)[2]/46,XY,t(3;19)(q12;q13)[3]/47, XY,t(3;19),t(4;8)(p16;p21),+20[6]/45,X,−Y[16]/46,X,−Y,+12,inv(12)(q13q24)[13]/46,XY,t(6;16;8)(p21;p13;p21)[45]/46,XY,t(3;5)(q25; q13)[20]/46,XY,der(1)inv(1)(p36q42)inv(1)(q25q42)[16]/46,XY,**del(1)(q12)**[12]/46,XY,del(6)(q21q23)[12]/46,XY,t(11;12)(q12;q24),t(17; 21)(q21;p11)[11]/46,XY,t(1;6)(q21;q21),inv(5)(p15q13)[8]/46,XY,t(6;10)(q21;q26)[8]/46,XY,inv(7)(p22q11)[8]/46,XY,t(3;21)(p21;p11), t(16;18)(p13;q21)[7]/46,XY,t(6;20)(p21;p13)[7]/46,X,t(Y;10)(q12;q24)[6]/46,XY,t(1;8)(p36;q24)[6]/46,XY,del(2)(q23q33),der(9)ins(9;2) (p23;q33q23)del(9)(p24)[6]/46,XY,t(7;9)(p13;q32)[6]/46,Y,t(X;4;14)(q21;q21;q32)[5]/46,XY,t(1;9)(p13;p13)[5]/46,XY,t(1;21)(p35; q22)[5]/46,XY,del(1)(q32)[5]/46,XY,add(1)(q44)[5]/47,XY,+7[4]/46,XY,t(7;7)(p22;q22)[4]/46,XY,t(8;10)(q24;q11)[4]/46,XY,t(1;3)(p11; q12)[3]/46,XY,del(1)(q25)[3]/46,XY,t(1;18)(q25;q11),inv(6)(p11p21)[3]/46,XY,add(2)(q37),der(11)t(11;12)(q23;q15),del(12)(q13)[3]/ 46,XY,t(4;6)(q31;p25)[3]/46,XY,t(5;13)(q13;q14)[3]/46,XY,t(5;13)(q33;q14)[3]/47,XY,del(11)(q23),+12[3]/46,XY,inv(12)(q21q24)[3]/ 46,XY,t(12;14)(q22;q32),inv(17)(q21q25)[3]/46,XY,add(20)(q13)[3]/46,Y,t(X;19)(q24;p13)[2]/46,X,t(Y;22)(q12;q11)[2]/46,XY,t(1; 10)(q21;p11)[2]/46,XY,tas(1;20)(q44;q13)[2]/46,XY,tas(1;21)(q44;p13)[2]/46,XY,tas(1;22)(q44;p13)[2]/46,XY,t(2;7)(q11;p22)[2]/ 46,XY,t(3;11)(p21;q23)[2]/46,XY,t(4;16)(p14;p13)[2]/46,XY,der(6)t(6;9)(q21;q34)inv(6)(p11q21),der(9)t(6;9)(q21;q34)[2]/46,XY, del(7)(p11)[2]/46,XY,t(7;10)(q11;p13)[2]/46,XY,t(8;10)(p23;q11)[2]/46,XY,**del(11)(p13)**[2]/46,XY,t(17;17)(q21;q25)[2]/46,XY,t(17; 19)(q21;p13)[2]/46,XY,r(18)[2]/46,XY[460]/nonclonal[112]	58 / 54:3+2+2+1×51 [903]
17/1707-93	56–59,X,−X,add(X)(q22),−1,+del(2)(p22),der(2)inv(2)(p13q11)t(2;20)(q11;q11),der(2)t(2;21)(q11;q11)×2,−3,der(3)add(3)(p25)del(3) (q12q21)del(3)(q21q23),**del(4)(q25)**,inv(4)(p16q27),der(5)t(5;8)(p13;q11),der(6)t(6;17)(p11;q21)del(6)(q21),der(6)del(6)(q23)t(6; 15)(p21;q22),der(6)del(6)(q23)t(6;19)(p22;q11),del(7)(q21),der(7;9)dic(7;9)(q22;p13)dup(7)(q36q22)add(9)(q34),−8,−9,−9,−10, add(10)(q26),−11,der(12)inv(12)(p11q13)del(12)(p11)del(12)(q21q24),der(12)t(3;?;12)(q11;?;q24)del(3)(q12q21)del(3)(q21q23),−13, **der(13;15)(q10;q10)**,der(13;20)(q10;q10),−14,der(14)t(9;14)(q21;p11),−15,−15,der(16)t(15;16)(q11;p13)del(15)(q12q21),der(16)t(3; 16)(q21;q24),−17,**del(18)(q12)**×2,+add(18)(q12),+der(18)t(3;18)(q11;q12)del(3)(q12q21)del(3)(q21q23),−19,der(19)t(4;19)(q21; p13)t(6;19)(p22;q11),der(20)t(11;20)(q13;q13),+der(20)t(12;20)(q15;q11)del(12)(q22q23)del(20)(p12),−21,−22,der(22)t(X;22)(q11; p11),+5mar[91]/105–121,idem×2[12]/47,XX,+7[6]/48,XX,+5,+7[6]/47,XX,+X[2]/46,XX[190]/nonclonal[7]	5 / 3:2+2+1 [314]
18/2238-93	75–86,X,i(X)(q10),Y,−Y,−1,add(2)(q33),**i(3)(q10)**×2,−4,−6,−6,t(6;15)(q21;q15)×2,der(7)t(6;7)(p21;p15)×2,+add(8)(q24),+add(8)(q24), del(10)(q23q24)×2,−13,−13,−17,der(17)r(17;17)(p11q25;q11q23),−18,−19,−21,−21,−22,i(22)(q10)×2,+der(?)t(?;6)(?;q15), +der(?)t(?;6)del(6)(q22),+mar1×2–3[113]/74–83,idem,+del(10)(q11)[2]/79–84,idem,+r[4]/83–86,idem,+der(22)t(8;22)(q11;p11)[4]/ 82–85,idem,+der(22)t(16;22)(q11;p13)[5]/150–172,idem×2[34]/45,X,−Y[11]/46,XY[36]/nonclonal[7]	7 / −:7 [216]

TABLE 2. Cytogenetic Findings in the Pancreatic Carcinomas (continued)

Case No./ Lab No.	Karyotype[a]	No. of abnormal clones unrelated: related (subclones) [No. of cells analyzed]
19/2378-93	76–82,XX,−Y,t(1;5)(p34;p15),+der(1)t(1;5),inv(2)(p25q21),−3,der(4)t(4;6)(q33;q23)×2,der(6)dup(6)(p21p22)add(6)(q13),der(6)del(6)(q13q15)add(6)(q23),der(6)t(2;6)(q31;p21)hsr(2)(q31)del(6)add(6)(q23),+7,+der(7)t(7;7)(p12;q31),add(8)(p21)×2,+der(8)t(3;8)(q11;p11),+t(8;19)(p11;q11),+der(8)t(8;19)×2,add(10)(q26),+der(10)t(1;10)(q12;q23),+add(11)(p11),−12,+13,t(15;15)(q21q26;q22q24),+der(16)t(16;21)(q11;q11),−18,−19,add(19)(q13),i(20)(q10),+der(20)i(20)(q10)add(20)(q13),−21,+4mar[51]/76–79,idem,**del(1)(q11)**[2]/74–76,idem,**i(8)(q10)**[2]/152–164,idem×2[11]/230–328,idem×3–4[2]/47,XY,+7[4]/46,XY[18]/nonclonal[1]	6 / −:6 [91]
20/2517-93	60-123,X,−X,−X,−1,i(2)(q10),**i(3)(q10)**,−4,−5,−6,del(6)(q23),+7,dic(7;19)(q22;p13),−9,+13,−17,−18,add(19)(p13),add(20)(p11),−21,+4mar,inc[5]/46,XX,t(13;18)(q11;p11)[3]/46,XX[44]/nonclonal[11]	2 / 2:1+1 [63]
21/2605-93	84–87,XX,−Y,−Y,−1,inv(1)(p34q44),+2,−3,del(5)(q13q33),der(5)t(5;8)(q11;q11),−6,add(6)(q15),+7,+7,−8,der(8)t(1;8)(q11;p12)inv(1),dic(9;22)(p22;p11)×3,del(9)(p22),+del(9)(q22),−10,+11,+11,+del(12)(q12q22),+der(12)del(12)t(9;12)(p11;p13)dic(9;22)add(22)(q13),der(13)t(3;13)(q11;q34),−15,−16,−17,−17,der(18)t(17;18)(q11;q12)×2,+19,add(19)(p11)×2,i(21)(q10)[32]/86–87,idem,dmin[6]/170–350,idem×2–4,dmin[cp13]/45,X,−Y[4]/46,XY[7]/nonclonal[2]	4 / −:4 [64]
22/630-94	46,XY,t(2;10)(p11;p13)[4]/46,XY,**del(1)(q21)**[2]/47,XY,+18[2]/46,XY[215]/nonclonal[5]	3 / 3:1+1+1 [228]
23/2913-92	45,X,−Y[22]/46,idem,+7[2]/45,idem,t(1;16;3)(q25;q24;p13)[12]/45,idem,inv(4)(p16q31),t(14;17)(q32;q11)[2]/45,idem,t(14;15)(q11;q24)[3]/46,XY,t(1;9)(p34;q34)[3]/47,XY,t(2;5)(p13;p15),+18[2]/46,XY[114]/nonclonal[4]	7 / 3:5+1+1 [164]
24/979-93	46,XX,t(1;2)(q23;p13)[2]/46,XX[126]/nonclonal[32]	1 [160]
25/2620-93	44-45,X,−Y,der(1;12)(q10;q10),+r(1)(p34q32),der(6)t(1;6)(q21;q25),dic(6;22)(p21;q13),+8,der(8;20)(q10;q10),add(9)(p22),del(11)(q14q23),+1–2mar[6]/44–45,idem,−dic(6;22),+der(6;22)dic(6;22)del(6)(q21)[3]/44–45,idem,−dic(6;22),+der(6;22)dic(6;22)hsr(6)(p21)[39]/88–90,idem×2,−dic(6;22)×2,+der(6;22)dic(6;22)hsr(6)×2[6]/46,XY[24]/nonclonal[6]	4 / −:4 [84]
26/1330-94[c]	38–39,XX,−3,der(4;14)(q10;q10),der(5)t(1;5)(q12;q35),**i(5)(p10)**,der(6)t(3;6)(q12;p22)ins(6;?)(p22;?),−9,der(14;21)(q10;q10),−15,−17,der(18)t(18;21)(q11;q11),der(19)t(5;19)(q22;q13),add(20)(q13),−21,der(22)t(17;22)(q11;p11),add(22)(q13)[8]/38,idem,+der(5;14)(p10;q10),−i(5)(p10),−der(14;21),−der(19)t(5;19),+der(19;21)(q10;q10)t(5;19)(q22;q13)[15]/77–79,idem×2,+der(5;14)×2,−i(5)(p10)×2,−der(14;21)×2[3]/74–76,idem×2,add(1)(q12)×2,+der(5)ins(5;19)(q11;q12q13)del(5)(q11q22)×2,−i(5)(p10)×2,−der(19)t(5;19)×2[50]/148–152,idem×4,add(1)×4, +der(5)ins(5;19)del(5)×4,−i(5)(p10)×4,−der(19)t(5;19)×4[20]/47,XX,+7[2]/46,XX,t(9;10)(p12;q23)[10]/47,XX,+X[3]/46,XX,**del(1)(q21)**[2]/46,XX,t(3;22)(q21;q13)[2]/46,XX[46]/nonclonal[6]	10 / 5:6+1+1+1+1 [167]
27/1258-94	46–48,XX,**del(3)(p11)**,+r[3]/45,X,−X[3]/46,XX,**del(6)(q15)**[2]/47,XX,+X[2]/46,XX[75]/nonclonal[22]	4 / −:4 [107]

TABLE 2. Cytogenetic Findings in the Pancreatic Carcinomas (continued)

Case No./ Lab No.	Karyotype[a]	No. of abnormal clones unrelated: related (subclones) [No. of cells analyzed]
28/129-94[c]	63–65,−X,del(X)(q21q21),−Y,−3,add(4)(q33),**i(5)(p10)**,−6,der(6)t(6;7)(p23;q11)×2,add(7)(q32)×2,der(7)t(7;18)(q11;q21),+der(7)t(7;18),+8,−9,−10,add(11)(q13),**i(12)(p10)**,−13,+14,der(14)add(14)(p11)add(14)(q24)×2,−15,−16,add(18)(q11)×2, **del(18)(q21)**, +del(18),der(19)t(19;20)(p12–13;p11),−21,+22,+mar[183]/63–65,idem,del(20)(p11)[3]/54,idem,der(4)t(4;17)(q21;q11)[2]/62–65, idem,der(4)add(4)(p11)t(4;20)(q35;q11),−20[55]/63–65,idem,der(4)add(4)t(4;20),**i(8)(q10)**,−20[4]/63,idem,der(4)add(4)t(4;20),der(8) t(8;17)(p11;q11),−17,−20[3]/63–65,idem,−15,der(20)t(15;20)(q11;p11)[14]/63,idem,−15,der(17)t(8;17)(q22;q23),der(20)t(15;20)[3]/ 126–130,idem×2[46]/126–130,idem×2,−15,−15,der(20)t(15;20)×2[4]/124–195,idem×2–3,der(4)add(4)t(4;20)×2–3,−20,−20[7]/180–330,idem×3–5[6]/45,X,−Y[26]/46,XY[118]/nonclonal[6]	13 −:13 [480]

[a]Recurrent aberrations are in bold type; each of them is marked only once per case.
[b]Previously published in Johansson et al. (1994).
[c]The identification of the chromosome 18 and 19 abnormalities in this case was also based on FISH analyses (Höglund et al., 1998a,b).
[d]Previously published in Johansson et al. (1994), but the karyotype has been revised.
[e]Previously published in Gorunova et al. (1995).

DISCUSSION

Only about 70 cytogenetically abnormal malignant exocrine pancreatic neoplasms have been reported (Mitelman, 1998). Furthermore, because of the complexity of aberrations and the suboptimal quality or quantity of metaphases, many karyotypes have included a high proportion of unidentified chromosomal material. The present study adds 25 aberrant tumors to the total karyotypic database on nonendocrine pancreatic carcinoma, including three tumors (cases 13, 26, and 28; Figs. 2, 1D and 1C) subsequently maintained as low-passage cell lines (LPC2p, LPC3p, and LPC1p, respectively), currently used for molecular analyses (see Mahlamäki et al., 1997; Höglund et al., 1998a,b).

In genetic research on pancreatic cancer, sampling poses a serious problem because the late diagnosis and the aggressive course considerably limit the availability of specimens. Reflecting this situation, our series comprised 20 primary and nine abdominal metastatic lesions. We attempted to overcome the shortage of samples by using needle biopsy specimens in two cases. Although only one of these yielded aberrant and high-quality chromosomes (Fig. 1B), such material may prove to be a valuable help in the cytogenetic analysis of pancreatic cancer. We also widened the spectrum of karyotypically studied epithelial pancreatic neoplasms by including, for example, papilla Vateri tumors and small cell carcinomas. The specific biologic and clinical features of such different tumor varieties (Klöppel and Maillet, 1989) notwithstanding, we chose to pool all data when delineating the karyotypic profile of nonendocrine pancreatic carcinoma. The reason for this was that there were too few cases per histologic subgroup to allow meaningful statistical comparisons.

One aspect not yet properly clarified in pancreatic carcinoma is the cytogenetic intratumor variability seen as karyotypically related or unrelated clones. Heterogeneity has been described in 16 previously reported tumors, with two to three related clones in 11 cases and two unrelated clones in five (Johansson et al., 1992; Bardi et al., 1993; Griffin et al., 1994, 1995). The present data, however, revealed a profound karyotypic diversity in this tumor type. Not only was the proportion of multiclonal carcinomas nearly 80%, but also the total number of identified clones as well as the number of clones per tumor were unusually large; up to 39 related and 54 unrelated clones were detected. The clones comprised from two to hundreds of cells; however, the clonal size in a short-

term culture may not accurately correspond to that in vivo because of different selective pressures.

The finding of a broad spectrum of cytogenetically related clones in a tumor (Fig. 2) reflects a multiplicity of evolutionary pathways during neoplastic development and fits well with the somatic mutation theory of carcinogenesis (Nowell, 1976). Whereas polyploidization as a common way of generating new evolutionary modifications has been recorded in pancreatic cancer (Bardi et al., 1993, 1994; Griffin et al., 1994, 1995), next to nothing is known about karyotypic intraclonal divergence within the same ploidy level. Our data provide some remarkable examples of tumors containing 21 and 22 distinct near-diploid cell populations (cases 13 and 4) or 9 and 13 near-triploid subclones (cases 15 and 13). Such subclones may exhibit quite disparate karyotypic patterns. For example, in case 6 the three near-triploid populations shared only 11 changes, including six structural aberrations, but differed in more than 25.

Karyotypically unrelated clones were found in about 40% of the tumors in this series. Their pathogenetic importance in pancreatic carcinoma, as well as in other epithelial neoplasms such as skin, breast, and head-and-neck tumors (Heim et al., 1988; Pandis et al., 1995; Jin et al., 1996), remains uncertain. These clones are usually near-diploid, carry simple numerical or structural, mostly balanced, aberrations, may be multiple, and are often found together with highly abnormal cell populations. It is unknown whether the cytogenetically unrelated clones harbor common submicroscopic alterations and also whether these clones are a part of the tumor parenchyma. If genetically disparate clones represent the tumor population, the observed karyotypic diversity would signify a polyclonal origin in a rather high proportion of pancreatic carcinomas. If, on the other hand, unrelated clones, be they of mesenchymal or epithelial origin, are not truly neoplastic, their consistent presence and potential interactions with the tumor cells would still merit elucidation.

In view of the technical limitations of the cytogenetic analysis and the fact that the tumors were not monitored over time, which, in practice, is possible only for chronic hematologic malignancies, the present data obviously underestimate the actual intratumor cytogenetic divergence. The study of karyotypic heterogeneity is not only of academic interest. The numerous genetically heterogeneous clonal subsets with variable abilities to grow, differentiate, and metastasize and with different responses to the immune system and to genotoxic therapies present a major challenge in clinical oncology (Vogelstein and Kinzler, 1993; Sporn, 1996).

Whereas most carcinomas in the present study displayed massively rearranged karyotypes, four tumors had no cytogenetic abnormalities and six exhibited only simple changes (cases 1, 3, 22–24, and 27). The finding of only normal metaphases in some carcinomas most probably indicates that the stromal and not the cancer cells divided in vitro. The significance of simple abnormalities should also be interpreted with some caution. Certain numerical changes, such as +7, -Y, and +X, have been observed repeatedly in nonneoplastic conditions (Johansson et al., 1993; Mitelman et al., 1997a). Furthermore, trisomy 7 in case 3 hardly represents a neoplasia-associated abnormality in the primary tumor because the metastasis sampled seven months later displayed highly complex aberrations without this anomaly.

Also, the degree of cytogenetic complexity seen was considerably higher than reported before in pancreatic cancer (Johansson et al., 1992; Bardi et al., 1993; Griffin et al., 1994, 1995). The reasons for this may be the generally higher chromosome resolution level as well as the more detailed analysis performed, and also the fact that our series included a larger proportion of poorly differentiated and metastatic neoplasms. Indeed, poorly differentiated tumors have been shown to be more frequent among karyotypically complex pancreatic carcinomas (Johansson et al., 1994). As regards metastases, only one cytogenetically abnormal case has been reported (Bardi et al., 1994). Although no firm conclusions can be drawn at present, the karyotypic complexity of metastases seems comparable to that of poorly differentiated tumors.

The karyotypic complexity and intratumor heterogeneity notwithstanding, a nonrandom pattern of chromosomal aberrations is now emerging in pancreatic carcinoma. Thus, apart from eight previously reported recurrent structural changes in this tumor type — del(1)(q12), del(1)(q21), i(1)(q10), del(4)(q21), i(5)(p10), del(6)(q15), i(8)(q10), and del(9)(p13) (Mitelman et al., 1997b) — 11 new ones were identified: del(1)(q11), del(3)(p11), i(3)(q10), del(4)(q25), del(11)(p13), dup(11)(q13q23), i(12)(p10), der(13;15) (q10;q10), del(18)(q12), del(18)(q21), and i(19)(q10).

The common consequences of the chromosome 1 changes are losses in 1p, which our data seem to narrow to 1p34-pter, and gain of 1q, repeatedly

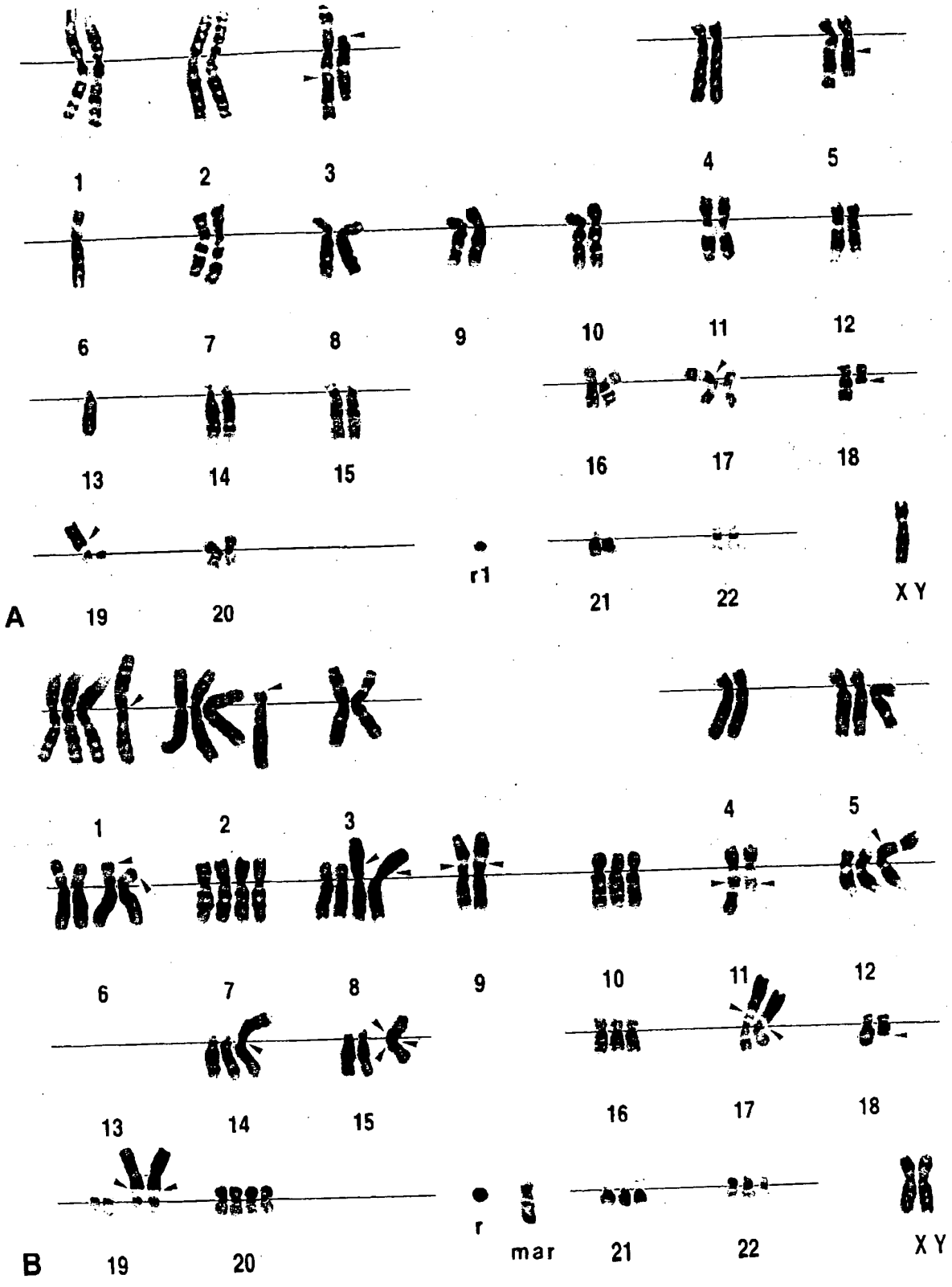


Figure 1. Representative karyograms of pancreatic carcinoma (for karyotypic description, see Table 2). **A**: Hypodiploid clone from case 4. **B**: Near-triploid clone from needle biopsy material in case 15. **C** and **D**: Near-triploid clones from tumors of origin for cell lines LPC1p and LPC3p, from cases 28 and 26, respectively. In **D**, extra copies of some chromosomes are nonclonal. Arrowheads indicate breakpoints.

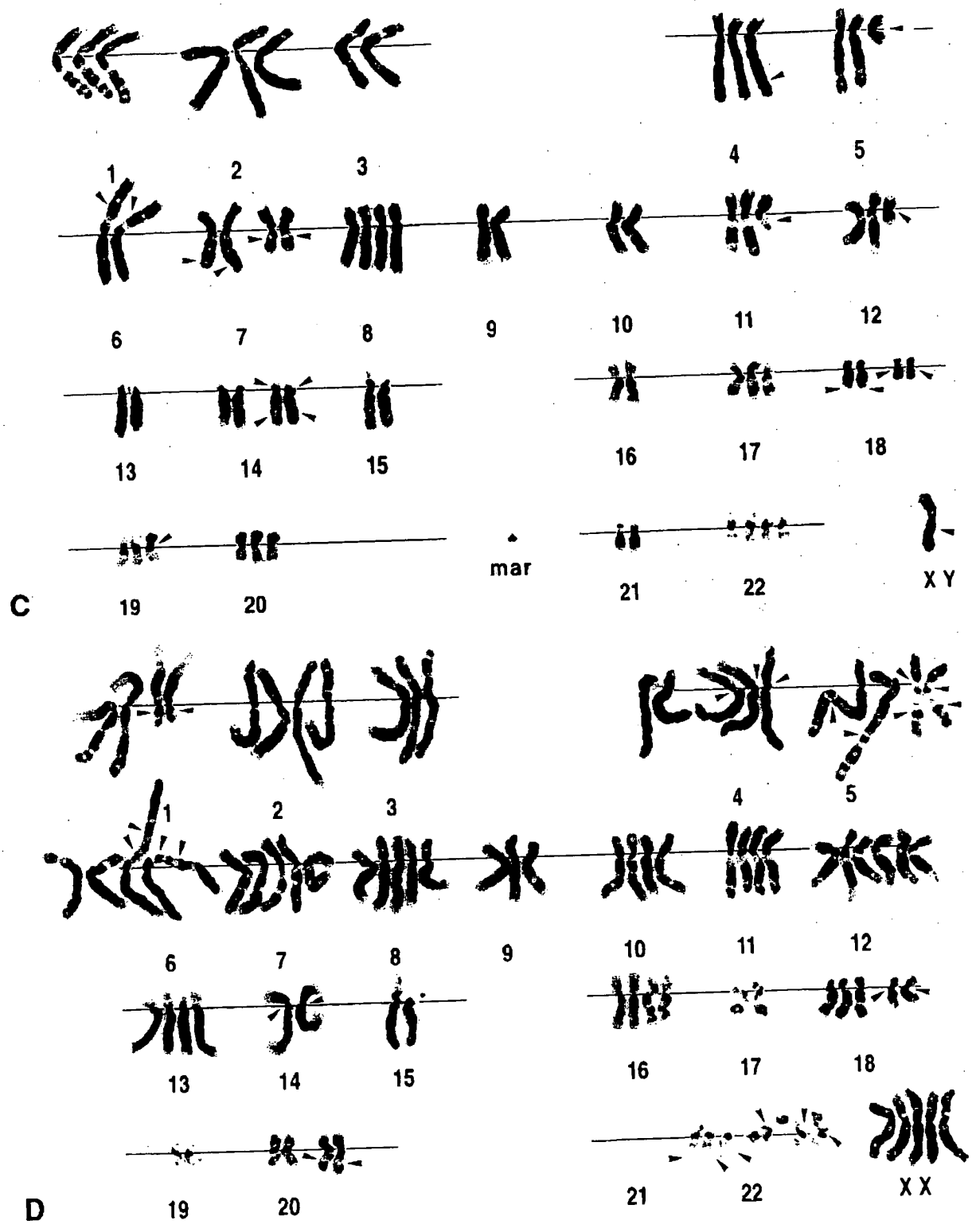


Fig. 1 (continued)

found as i(1)(q10) (Bardi et al., 1993; Griffin et al., 1995). The opposite outcome, loss in 1q, was also seen as recurrent del(1)(q11), del(1)(q12), and del(1)(q21). LOH analyses have confirmed frequent losses in 1p in pancreatic tumors (Hahn et al., 1995; Kimura et al., 1996); however, the essential molecular outcome of these imbalances is unknown (Schwab et al., 1996).

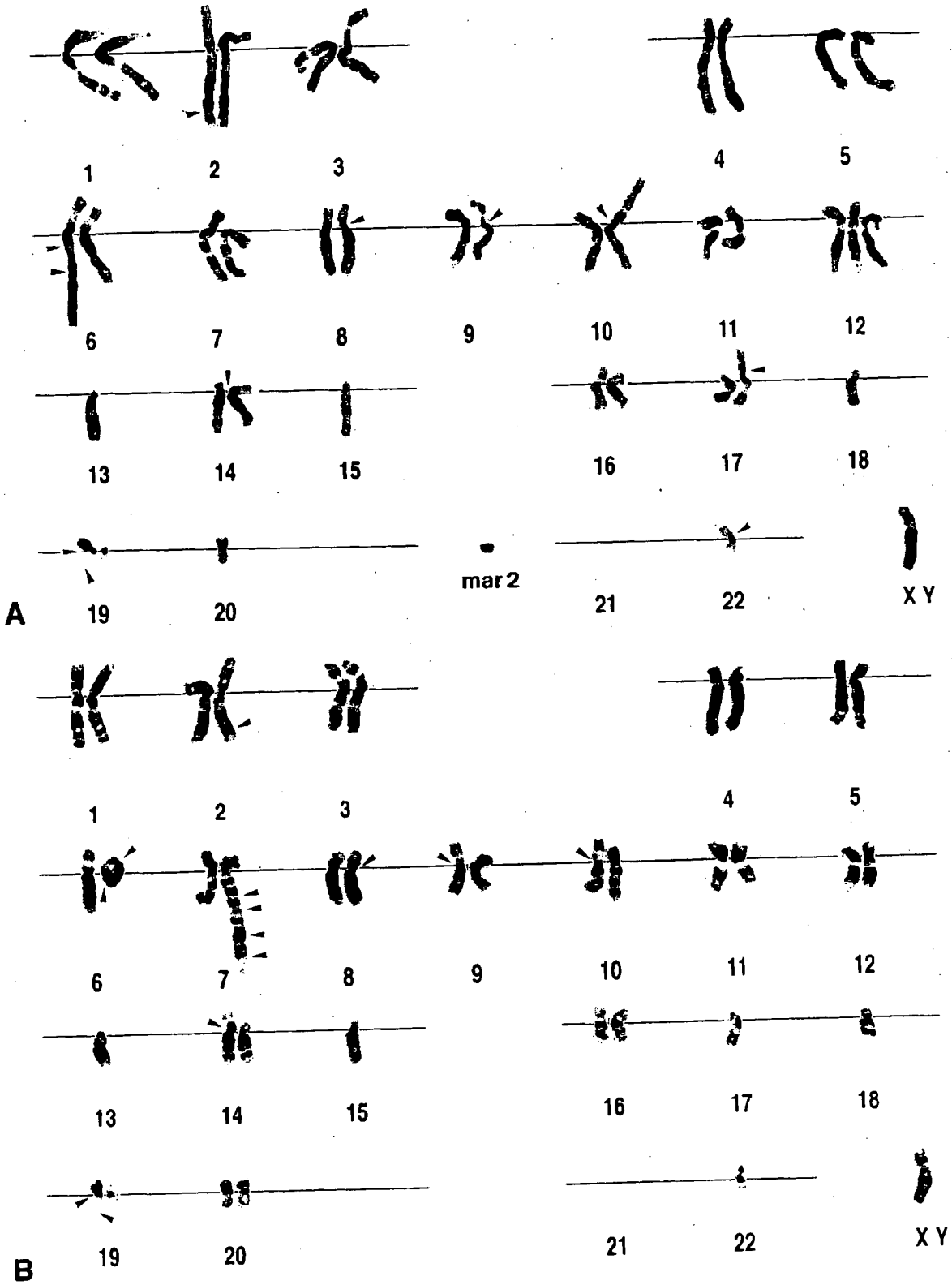
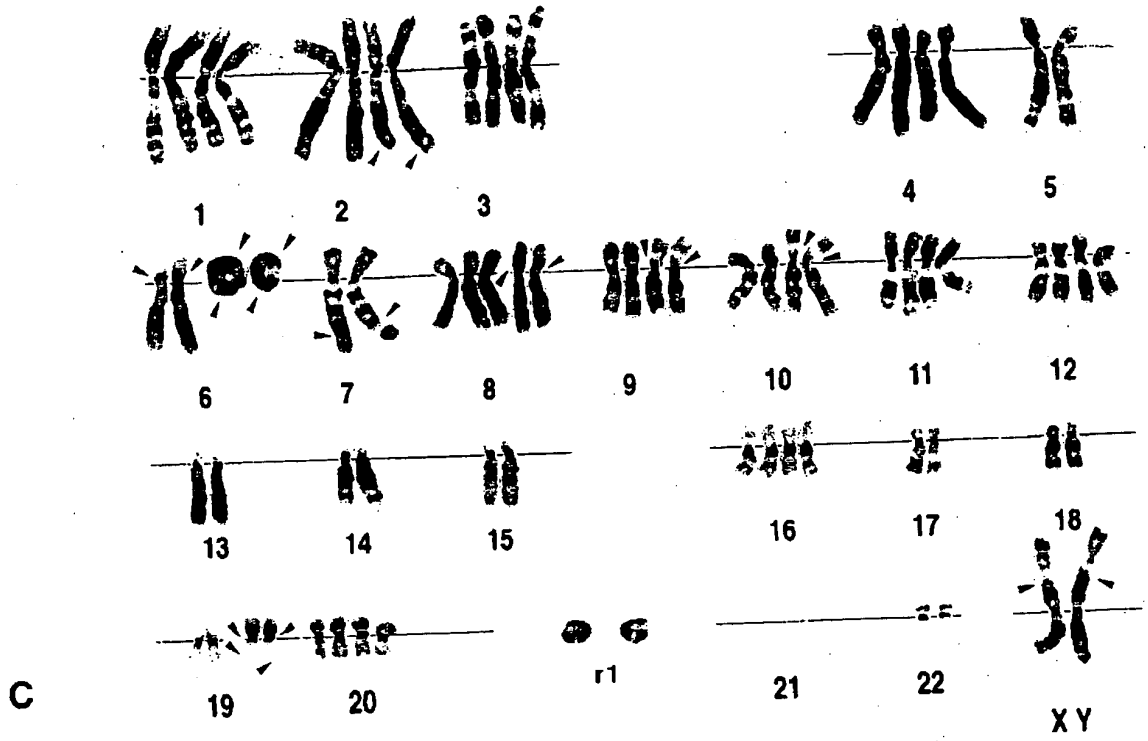
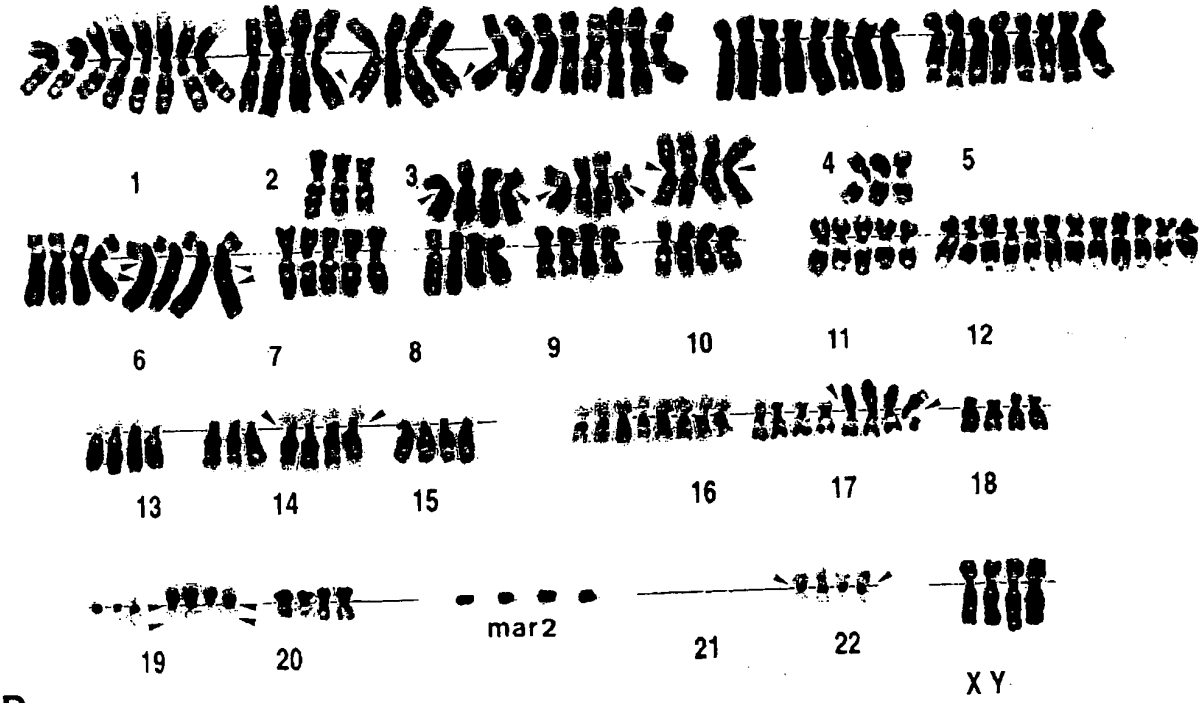


Figure 2. Representative karyograms illustrating clonal evolution in case 13 (tumor of origin for LPC2p cell line). **A** and **B**: Hypodiploid clones. **C**: Near-triploid clone. **D**: Near-hexaploid clone. Arrowheads indicate breakpoints.



C



D

Fig. 2 (continued)

Compared with the reported data on rearrangements affecting chromosome 3, particularly 3p, in pancreatic carcinoma (Bardi et al., 1993; Griffin et al., 1994, 1995), our results highlight the near-centromeric region 3p11-q12 as the clustering site of more than 50% of the breakpoints. A clear-cut pattern of imbalances was identified as loss of 3p and gain of 3q, with the recurrent changes

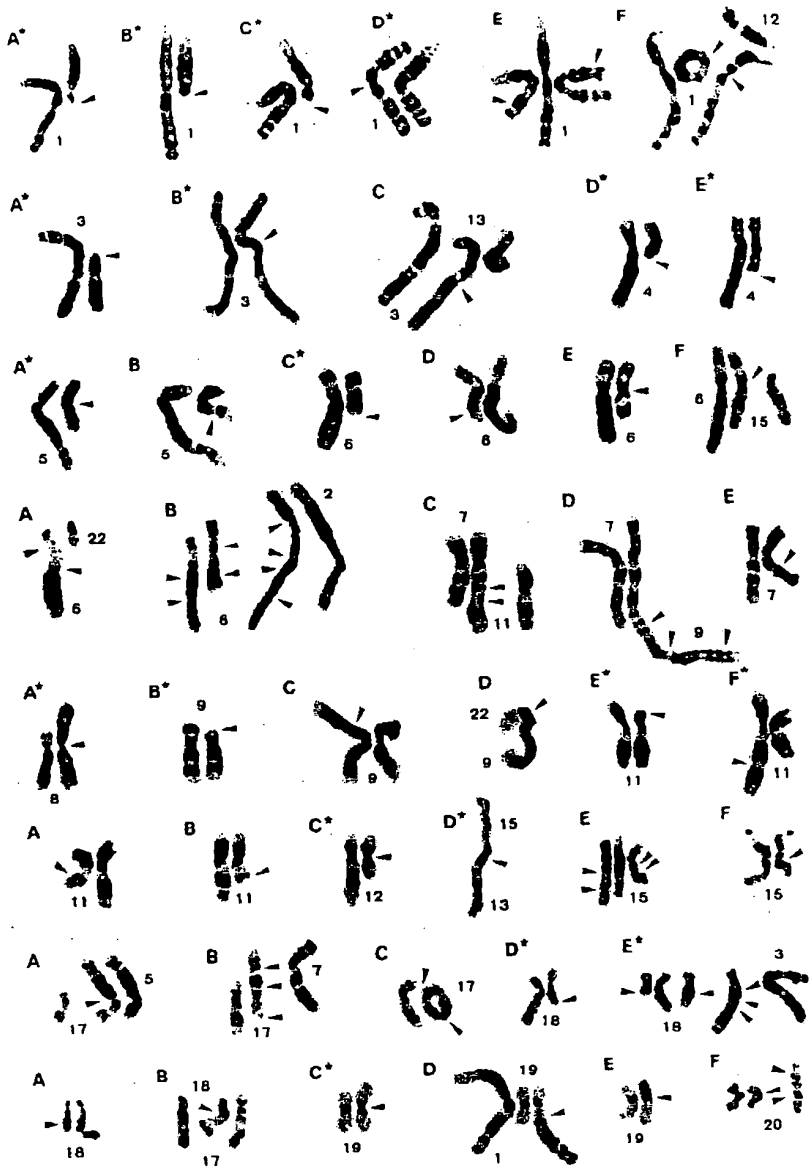


Figure 3. Recurrent aberrations and chromosomal rearrangements resulting in characteristic karyotypic imbalances in pancreatic carcinoma. Normal chromosomes, when present in the same metaphase, are also shown. From top to bottom, 1st row. **A**: Case 19, del(1)(q11). **B**: Case 15, del(1)(q12). **C**: Case 22, del(1)(q21). **D**: Case 6, i(1)(q10). **E**: Case 7, add(1)(q25), add(1)(p34). **F**: Case 25, r(1), der(1;12)(q10;q10). 2nd row. **A**: Case 9, del(3)(p11). **B**: Case 18, i(3)(q10). **C**: Case 21, der(13)t(3;13). **D**: Case 13, del(4)(q21). **E**: Case 17, del(4)(q25). 3rd row. **A**: Case 28, i(5)(p10). **B**: Case 21, del(5)(q13q33). **C**: Case 12, del(6)(q15). **D**: Case 21, add(6)(q15). **E**: Case 6, i(6)(p10). **F**: Case 9, der(15)t(6;15). 4th row. **A**: Case 25, der(6;22)dic(6;22)hsr(6). **B**: Case 19, der(6)del(6)add(6), der(6)dup(6)add(6), der(6)t(2;6)hsr(2) del(6)add(6). **C**: Case 6, der(7)t(7;11)dup(11). **D**: Case 17, der(7;9)dic (7;9)dup(7)add(9). **E**: Case 19, der(7)t(7;7). 5th row. **A**: Case 28, i(8)(q10). **B**: Case 6, del(9)(p13). **C**: Case 25, add(9)(p22). **D**: Case 21, dic(9;22)(p22;p11). **E**: Case 10, del(11)(p13). **F**: Case 13, dup(11)(q13q23). 6th row. **A**: Case 25, del(11)(q14q23). **B**: Case 6, del(11)(q21). **C**: Case 28, i(12)(q10). **D**: Case 17, der(13;15)(q10;q10). **E**: Case 19, t(15;15). **F**: Case 7, del(15)(q22q24). 7th row. **A**: Case 7, der(17)t(5;17). **B**: Case 9, der(17)inv(17)ins(17;7). **C**: Case 18, der(17)r(17;17). **D**: Case 15, del(18)(q21). **E**: Case 17, del(18)(q12), add(18)(q12), der(18)t(3;18)del(3)del(3). 8th row. **A**: Case 7, add(18)(q12). **B**: Case 21, der(18)t(17;18). **C**: Case 6, i(19)(q10). **D**: Case 9, der(19)t(1;19). **E**: Case 21, add(19)(p11). **F**: Case 19, i(20)(q10), i(20)(q10)add(20). Detailed karyotypic descriptions are given in Table 2. Asterisks denote recurrent changes and arrowheads indicate breakpoints.

del(3)(p11) and i(3)(q10). The frequent losses of 3p are in line with LOH (Seymour et al., 1994; Hahn et al., 1995) and CGH studies (Fukushige et al., 1997; Mahlamäki et al., 1997). The molecular basis of the chromosome 3 changes is unclear, except for a possible role of the *FHIT* gene at 3p14.2 as a TSG in different carcinomas, including pancreatic cancer (Ohta et al., 1996; Shridhar et al., 1996). The importance of *FHIT* in neoplasms has, however, recently been questioned (Panagopoulos et al., 1997; van den Berg et al., 1997), and the significance of *FHIT* alterations in various tumors remains to be elucidated.

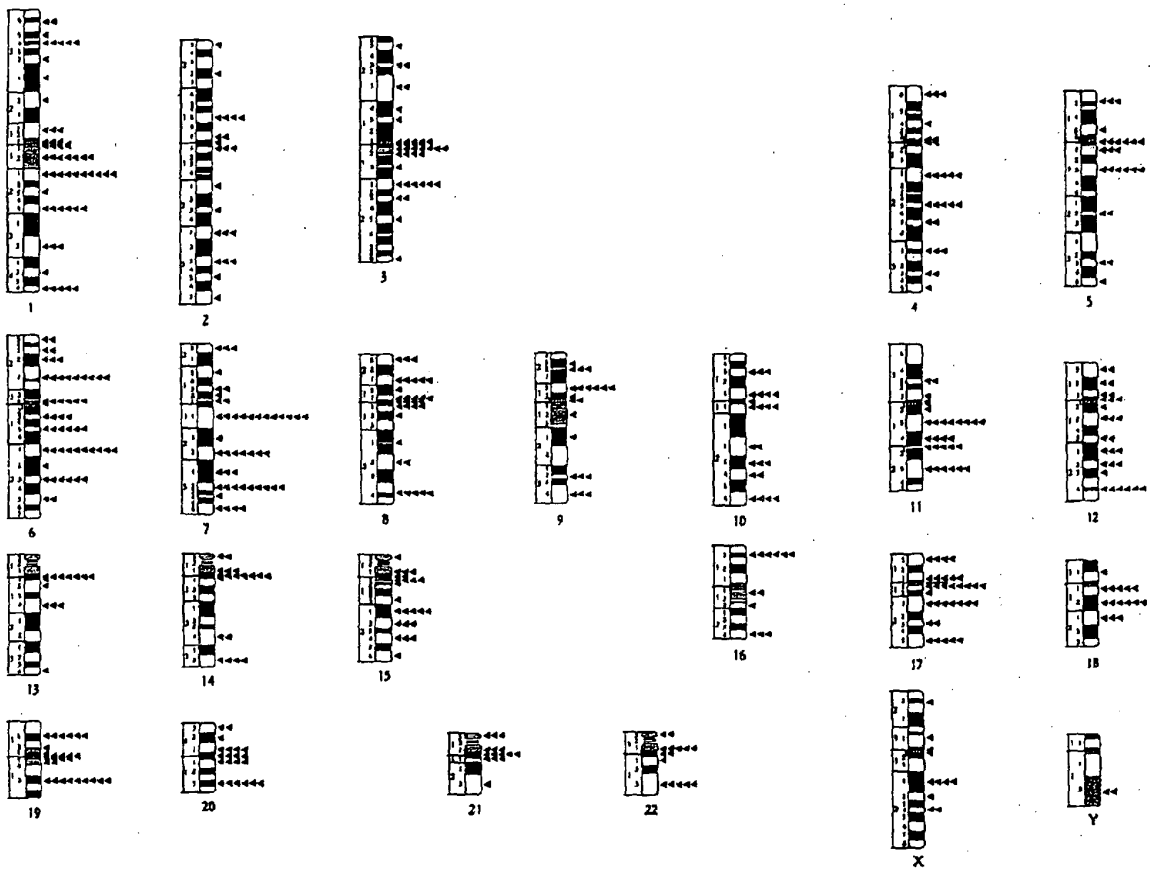


Figure 4. Distribution of the 608 breakpoints in structural chromosomal aberrations in 23 pancreatic carcinomas. See text for details.

We also detected a more frequent participation of chromosome 5 in structural rearrangements than shown before (Bardi et al., 1994; Griffin et al., 1995). The observed distinct overrepresentation of 5p, recurrently present as i(5)(p10), is supported by a recent CGH study (Fukushige et al., 1997).

The finding of frequent losses of 6q, with breakpoints distributed along the entire arm and a recurrent del(6)(q15), agrees well with previous cytogenetic (Bardi et al., 1994; Griffin et al., 1995) and molecular genetic (Hahn et al., 1995; Kimura et al., 1996; Fukushige et al., 1997) data. So far, no candidate TSG in 6q has been identified. The observed overrepresentation of 6p11–21 may suggest involvement of some dominant oncogenes. In this context, cases 19 and 25 with hsr in 6p21 (although the origin of the hsr is unknown) and case 26, in which high-level gains of 6cen-p21 were detected by CGH (Mahlamäki et al., 1997), may be of particular interest.

Apart from confirming frequent gains of chromosome 7 (Bardi et al., 1994; Griffin et al., 1995), our data delineate 7q11–32 as a commonly overrepresented region, with the minimal segment 7q11–22. CGH high-level increases in 7q22 (Solinas-Toldo et al., 1996) or in 7cen-q31 (Mahlamäki et al., 1997) are thus in accordance with these results. The present data provide further support for losses in 7q31-ter (Griffin et al., 1994). It is noteworthy that the cationic trypsinogen gene, mapping to 7q35, was recently shown to be mutated in hereditary pancreatitis, predisposing to pancreatic cancer (Whitcomb et al., 1996a,b).

The imbalance profile of chromosome 8 emphasizes the nonrandomness of 8p losses and 8q gains, with recurrent i(8)(q10), in pancreatic carcinoma (Bardi et al., 1994; Griffin et al., 1995; Hahn et al., 1995; Fukushige et al., 1997). The recently shown high-level increases in 8q23-qter (Mahlamäki et al., 1997) may have narrowed the critical region.

In contrast to the previously noted low frequency of cytogenetically recognized abnormalities in 9p (Bardi et al., 1994; Griffin et al., 1995; Brat et al., 1997), our data provide more karyotypic evidence of 9p losses, seen recurrently as del(9)(p13). The frequent occurrence of 9p involvement is also well

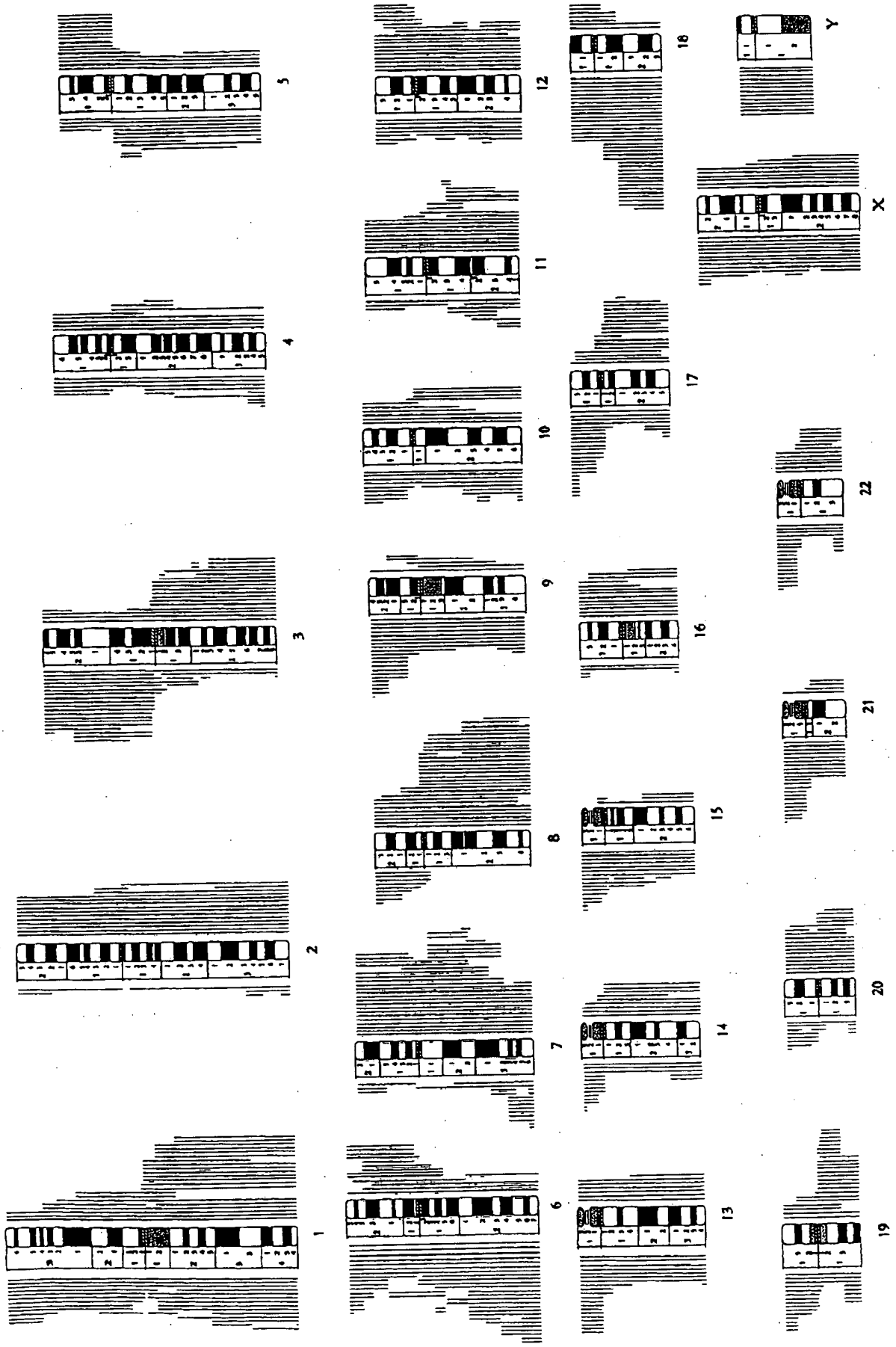


Figure 5. Karyotypic imbalances caused by numerical and structural aberrations in 25 pancreatic carcinomas. Losses are to the left, gains to the right. See text for details.

documented by molecular methods (Hahn et al., 1995; Solinas-Toldo et al., 1996; Fukushige et al., 1997; Mahlamäki et al., 1997). One target may well be *CDKN2A* at 9p21, shown to be inactivated in a large proportion of pancreatic tumors (Caldas et al., 1994; Bartsch et al., 1995).

In addition to gains of chromosome 11 reported earlier (Bardi et al., 1994; Griffin et al., 1995), our imbalance map depicted 11q11–13 as a commonly overrepresented segment, corresponding well to CGH gains in 11q, particularly in 11q13 (Fukushige et al., 1997; Mahlamäki et al., 1997). A possible pathogenetic consequence could be deregulation of cyclin D1 (Schuuring, 1995), and, in fact, *CCND1* overexpression was recently shown in pancreatic cancer (Gansauge et al., 1997).

Our karyotypic database provides a disparate interpretation of the chromosome 12 imbalances (cf. Bardi et al., 1994; Griffin et al., 1995), demonstrating gain, rather than loss, of this chromosome, especially 12p, repeatedly present as i(12)(p10). This finding, corroborated by CGH (Mahlamäki et al., 1997), may suggest activating alterations of, for example, *KRAS2* at 12p12, which is known to be mutated in most pancreatic neoplasms (Hruban et al., 1993).

Alterations within the *BRCA2* region in 13q12 (Goggins et al., 1996) and also allelic losses at 13q14, the site of *RB1* (Ruggeri et al., 1992), have been described in pancreatic carcinoma. Cytogenetic abnormalities of chromosome 13, however, have mainly been whole-copy losses (Griffin et al., 1995; Brat et al., 1997), and the same changes were found in our cases; only occasionally were losses at 13q12 and 13q14 seen. Thus, because CGH studies also detect deletions in 13q (Solinas-Toldo et al., 1996; Mahlamäki et al., 1997), the chromosomal basis of these losses still needs clarification.

The cytogenetic underrepresentation of chromosome 15, both as entire-copy losses and partial losses in the long arm, mostly in 15q11–22, which were detected for the first time in this study, is further supported by a very recent CGH analysis (Mahlamäki et al., 1997). The mapping of two genes, *SMAD3* and *SMAD6*, related to the recently identified TSG *SMAD4* at 18q21, to 15q21–22 (Riggins et al., 1996) makes this region attractive for detailed molecular examination.

The present results reveal a higher rate of structural aberrations and fewer numerical changes affecting chromosome 17 than reported previously. The common imbalance identified, loss of 17p (Bardi et al., 1994; Seymour et al., 1994; Griffin et al., 1995; Hahn et al., 1995; Kimura et al., 1996), presumably targets *TP53* at 17p13 (Ruggeri et al., 1992; Scarpa et al., 1993). The possible molecular basis of the observed gain of 17q (Bardi et al., 1994; Solinas-Toldo et al., 1996), which our data appear to limit to 17q11–23, has, on the other hand, not been investigated in pancreatic tumors.

Chromosome 18 abnormalities were reported in 50% of pancreatic carcinomas, predominantly as entire-copy losses (Bardi et al., 1994; Griffin et al., 1995). In contrast, we identified chromosome 18 aberrations in 80% of all of the tumors and in all but one karyotypically complex carcinoma. In addition to numerical changes, about 50% of the tumors exhibited structural, sometimes multiple, abnormalities of this chromosome. Apart from trisomy 18 in two karyotypically simple clones, the essential imbalances were loss of chromosome 18 or 18q, with recurrent del(18)(q12) and del(18)(q21), and gain of 18p. Most of the breaks occurred in 18q11–12, with the minimal deleted region 18q21-qter. A high frequency of 18q losses has been shown by LOH (Seymour et al., 1994; Hahn et al., 1995; Kimura et al., 1996), CGH (Solinas-Toldo et al., 1996; Fukushige et al., 1997; Mahlamäki et al., 1997), and FISH (Höglund et al., 1998b) analyses; 18p overrepresentation has also been detected by the latter two techniques. Two possible deletion targets at 18q21 are strong candidate TSGs in pancreatic carcinoma—*SMAD4*, frequently inactivated by mutations and homozygous deletions (Hahn et al., 1996; Schutte et al., 1996), and *DCC* (Höhne et al., 1992). However, the association of *DCC* with neoplasia has recently been questioned (Fazeli et al., 1997). Other putative TSGs on 18q may contribute to pancreatic carcinogenesis, for example, *SMAD2* (formerly *MADR2*), recently mapped near *SMAD4* (Eppert et al., 1996; Uchida et al., 1996). Furthermore, the near-centromeric clustering of breakpoints at 18q may suggest involvement of additional critical genes (Höglund et al., 1998b).

Whereas the previous cytogenetic studies did not reveal any consistent pattern of chromosome 19 imbalances in pancreatic cancer, our karyotypic data demonstrate frequent underrepresentation of 19p, with the minimal region 19p13-pter, and overrepresentation of 19q, with the recurrent aberration i(19)(q10) and the commonly gained segment 19cen-q13. A recent FISH study (Höglund et al., 1998a) has confirmed and extended these results, limiting the region of loss to 19p13.3 and gain to 19q13.1–2. The overrepresentation of 19q may target *AKT2*, shown to be amplified and overex-

pressed in this tumor type (Cheng et al., 1996; Miwa et al., 1996).

In addition to +20 (Bardi et al., 1994; Griffin et al., 1995), we also found frequent structural changes of this chromosome, often involving the near-centromeric region. The essential outcome of these alterations seems to be gain of 20q, which has been corroborated by CGH analyses (Solinas-Toldo et al., 1996; Fukushige et al., 1997; Mahlamäki et al., 1997), and loss of 20p.

Finally, the imbalance map indicates that nonrandom gains are no less significant than losses in the karyotypic profile of pancreatic carcinoma. Similar conclusions have been reached in CGH studies (Solinas-Toldo et al., 1996; Fukushige et al., 1997; Mahlamäki et al., 1997). It is of importance in this context that the clonal evolution data from our own and previous analyses (Bardi et al., 1993; Griffin et al., 1994, 1995) indicate that different steps of karyotypic progression are associated with different imbalance patterns. At the hypodiploid level, characterized by generally less complex karyotypes, partial and entire-copy losses prevail over gains (Figs. 1A, 2A,B), whereas chromosomal gains become prominent after one or more rounds of polyploidization (Figs. 2C,D).

In conclusion, the karyotypic profile of pancreatic carcinoma fits the multistep carcinogenesis concept. The observed cytogenetic heterogeneity appears to reflect a multitude of interchangeable but oncogenetically equivalent events, and the nonrandomness of the chromosomal alterations underscores the preferential pathways involved in tumor initiation and progression.

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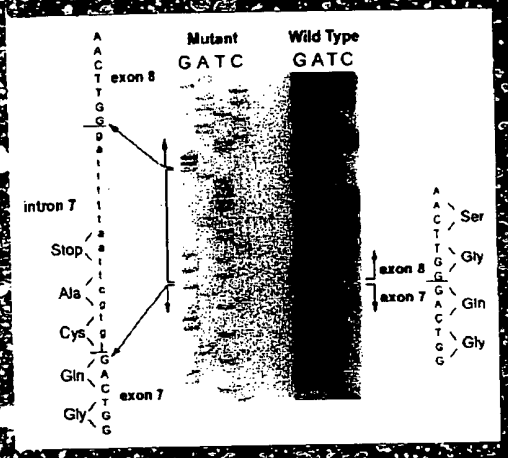

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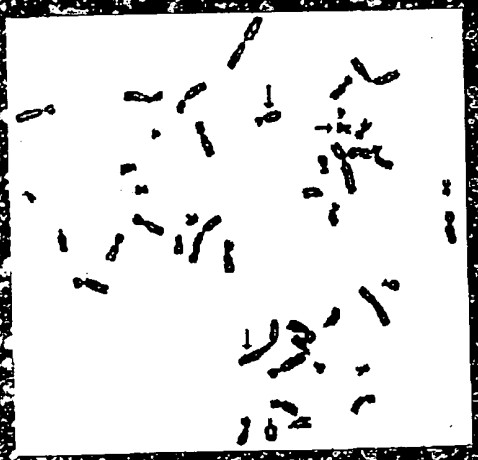

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Thomas A. WAGNER, et al.
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Thomas WAGNER, *et al.*

Title: HYBRID CELLS

Appl. No.: 09/756,293

Filing Date: January 9, 2001

Examiner: Q. Li

Art Unit: 1632

DECLARATION UNDER 37 C.F.R. § 1.132

I, Thomas E. Wagner, Ph.D., of 104 Golden Wings Way, Greer, South Carolina 29650, declare that:

1. I am employed as the Director of the Oncology Research Institute at Greenville Hospital. I have worked for more than 35 years in biomedical research, including oncology research. Attached is my *curriculum vitae* as Exhibit A.

2. I have supervised in the performance of the experiments detailed in Exhibit B to study the ability of hybrid cells to retain all antigens carried by tumor cells (Experiment I) and the loss of antigen over time by cultured cells carrying that antigen over time (Experiment II).

Experiment I

B16F0/hIL-2 cells were stained with red dye and fused with denddritic cells stained with green dye by PEG. Hybrid cells sorted out by FACS were cytospan on slides and immunocytochemically stained for the antigen (human IL-2).

Figure A shows the isotype control.

Figure B shows anti-human IL-2 antibody staining.

The data presented in Figures A and B shows that all the hybrid cells are human IL-2 positive. These results confirm that hybrid cells are capable of retaining all antigens carried by the original population of tumor cells.

Experiment II

In order to test the concept that specific antigens and/or particular type of cells in a cell mixture such as fresh tumor cell preparation can be lost during long time culture, B16F0/GFP tumor cells were either cultured alone or mixed with B16F0 tumor cells in a ratio of 1:1. The GFP positive cells were then monitored by fluorescent activated cell sorter (FACS) and plotted against time.

Figure A is a graph showing culture of B16F0/GFP tumor cells over a 20 day period. Because there is no selective pressure for the GFP marker protein, the GFP marker protein disappears from the cultured cells over the time period observed.

Figure B is a graph showing culture of a 1:1 ratio of B16F0/GFP tumor cells:B16F0 tumor cells over a 20 day period. Over time, the GFP marker protein is lost from cells and the culture is more homogeneous in cells lacking the GFP marker.

The data presented in Figures A and B show that antigen is lost during culture and cells carrying particular antigens can be lost during culture. These results confirm the theory that tumor antigen diversity of the original population of tumor cells is lost during long culturing periods. For example, after 5-10 days of culturing, the population of cells expressing the GFP antigen decreased from 50% to about 20%. These results suggest that if GFP-expressing cells had comprised a minor portion of the cell population (e.g., 2%), after 5 to 10 days in culture, GFP-expressing cells would no longer comprise a detectible portion of the cell culture.

3. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 10/02/03

Thomas E. Wagner, Ph.D.
Greenville Hospital

Curriculum Vitae

Thomas Edwards Wagner

Date of Birth: November 29

Education:

B.A. Princeton University, Magna Cum Laude, 1964 (Chemistry &Biology)
Ph.D. Northwestern University, 1966 (Biochemistry)

Employment:

Assistant Professor/Postdoctoral Associate
Wellsley College/Harvard.....1966-1968

Assistant Professor/Associate Member
Cornell University College of Medicine/Sloan-Kettering Institute
for Cancer Research..... 1968-1970

Assistant Professor of Biochemistry
Ohio University..... 1970-1972

Associate Professor of Biochemistry
Ohio University..... 1972-1975

Professor of Biochemistry
Ohio University..... 1975-1993

Visiting Research Faculty
Cambridge University..... 1982-1983

Professor & Chairman, Department Molecular & Cellular Biology
Ohio University..... 1983-1989

Founder and Director, Edison Biotechnology Institute
Ohio University..... 1984-1990

Distinguished Professor of Molecular & Cellular Biology

Ohio University..... 1993-1998

Greenville Hospital System.....1998-Present
Director of Oncology Research Institute

Distinguished Professor of Molecular & Cellular Biology
Clemson University.....1998-Present

Consultancies and Nati nal Committees:

Member, Combined RAC/ABRAC committee for the oversight and regulation of recombinant DNA experimentation, 1988-1990.

Consultant to the States of Ohio, Louisiana, North Carolina and New York for the development of State funded Biotechnology Programs, 1985 -1990.

Key Consultant, UN Biotechnology Development Project in India, 1989-1992.

Member, Office of Technology Assessment, Committee on Biotechnology, US Congress, 1986-1989.

Member, US National Research Council committees on "Improving the Nutritional Attributes of Food"1986-1988, and "Managing Global Genetic Resources" 1988-1991.

Advisor/Consultant to the office of the Presidential Science Advisor (President Regan) for biotechnology reporting to Dr. Beradine Healey 1986.

Consultant to Calgene, Inc., Davis California, Molecular Biology/Gene Delivery, 1984-1990.

Consultant and Member, Scientific Advisory Committee, GalaGen, Inc., Minneapolis Minnesota, 1991-present.

Senior Examiner and Consultant, Research Grants Council, Hong Kong, China, 1996-present.

Advisor and Honorary Professor of Molecular Biology, Tsing Hua University, Beijing China, 1997-present.

Honorary Professor of Biology, National Hydrobiology Institute, Chinese Academy of Sciences, Wuhan, China, 1997-present.

Member, American Association for Cancer Research (AACR) 1983- Present

Biotechnology Companies Founded and based upon discoveries made in my laboratory:

Diagnostic Hybrids Inc., Athens, Ohio, Founded 1982.
Medical Diagnostics.

DNX, Inc. [NADSAQ: DNXX], Princeton, N.J., Founded 1985.
Transgenic Technology.

Progenitor, Inc. [NASDAQ: PGEN], Columbus, Ohio, Founded 1992.
Gene and Cellular Therapeutics for Cancer and Immune Restoration.

Key Lectures Presented:

Opening plenary lecture at the first gene therapy conference, "NIH Public Forum on Human Gene Therapy" 1983.

Distinguished Lectureship in Biological Sciences, University of Tennessee, 1984.

Sir John Hammond Lecture, British Society of Biology, 1986.

Distinguished Lecture in the Life Sciences, Boyce Thompson Institute, Cornell University, 1987.

Distinguished Lecture, "Cancer Gene Therapy", The Hipple Cancer Center, 1994.

Centennial Lecture, " Biology and Medicine", The Rofant Society, Cleveland, Ohio, 1997.

Grant Funding:

During my tenure at Ohio University I have received grant support from the NIH Institutes of Child Health and Human Development and Allergy and Infectious Disease (AIDS), the National Science Foundation, the American Cancer Society, the State of Ohio Department of Development biotechnology fund, and several major and smaller pharmaceutical companies now totaling over $16 million.

Grants Received 1997-1998:

"Myocardial Hypertrophy and Heart Disease", N.I.H. $1,341,798.
"Embryonic Development", Progenitor, Inc., $430,765.
"SCID-hu Xenogenic Systems to Assay the Immune Maturation of Human Cord Blood Lymphocytes", Ross Laboratories, $ 400,000.
"Production of Genetically Engineered Peptides", Huagen. Inc., $932,827.
"Transgenic Services", NIH collaborative, $42,000.

Endowment Based Funding 1998-
"Oncology Research Endowment", GHS Foundation $10,000,000

Patents:

Genetic Transformation of Zygotes, US Patent No. 4,873,191, Granted Oct. 10, 1989, Thomas E. Wagner and Peter C. Hoppe (patent for the method of production of Transgenic Animals).

Gene Transfer Using Neodetermined Embryonic Cells, US Patent No. 5,032,407 Granted July 16, 1991, Thomas E. Wagner, Michael A. Reed and Barbara J. Corn (patent for the use of early lineage committed embryonic stem cells for cell-based gene therapy).

Virus-Resistant Transgenic Mice, US Patent No. 5,175,385, Granted Dec. 29, 1992, Thomas E. Wagner and Xiao-Zhou Chen (patent for a means of producing a virus resistant laboratory mouse).

DNA Polymerase Gene Expression system Utilizing an RNA Polymerase Co-Delivered with the Gene Expression Vector System, US Patent No. 5,591,601, Granted Jan. 7, 1997, Thomas E. Wagner, Xiaozhuo Chen and Yunsheng Li (patent for a novel cytoplasmic gene therapy vector).

Yolk-sac Cells and their Uses, Application No. 08/223,902, Notice of Allowance Dec. 11, 1997, Thomas E. Wagner and Michael R. Antczak (patent for the method of using Yolk-sac cells for transplantation therapy).

Method For Eliciting an Immune Responce Using a Gene Expression System that Co-Delivers an RNA Polymerase with DNA. Application No. 08/943,599, Notice of Allowance February 26, 1998, Thomas E. Wagner, Xiaozhuo Chen and Yunsheng Li (patent for a novel gene therapy vector for vaccination).

Transgenic Model of Heart Failure, U.S. S.N. 08/627,788 Filed March 1996, Notice of Allowance June 28, 2001, Thomas E. Wagner, Charles Homcy and Stephen Vatner. (patent for a transgenic mouse model of human heart failure).

Compositions and Methods for the use of Ribozymes to Determine Gene Function, US Patent No. 6,355,415, Granted March 12, 2002, Thomas E. Wagner and Yuefeng Xie.

Twenty four additional patent applications have been filed and are under processing with the US patent office covering a wide range of gene therapy systems, vectors and tumor therapies.

Publications (243):

Key Publication:

Wagner, T.E., Hoppe, P.C., Jollick, J.D., Scholl, D.R., Hodinka, R.L. and Gault, J.B.: Microinjection of a rabbit ß-globin gene into zygotes and its subsequent expression in adult mice and their offspring. Proc. Natl. Acad. Sci. USA 78, 6376-6380 (1981).

Publications over the past 10 years:

McNab AR, Andrus P, Wagner TE, Buhl AE, Waldon DJ, Kawabe TT, Rea TJ, Groppi V, Vogeli G. Hair-specific expression of chloramphenicol acetyltransferase in transgenic mice under the control of an ultra-high-sulfur keratin promoter. Proc Natl Acad Sci USA 1990 Sep;87(17):6848-52.

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A Human Prolactin Antagonist Modulates Transforming Growth Factors Beta-1 and Alpha in Human Breast Cancer Cells Ramamoorthy, P., Wagner, T.E. and Chen,W.Y. AACR 2000.

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Research Focus:

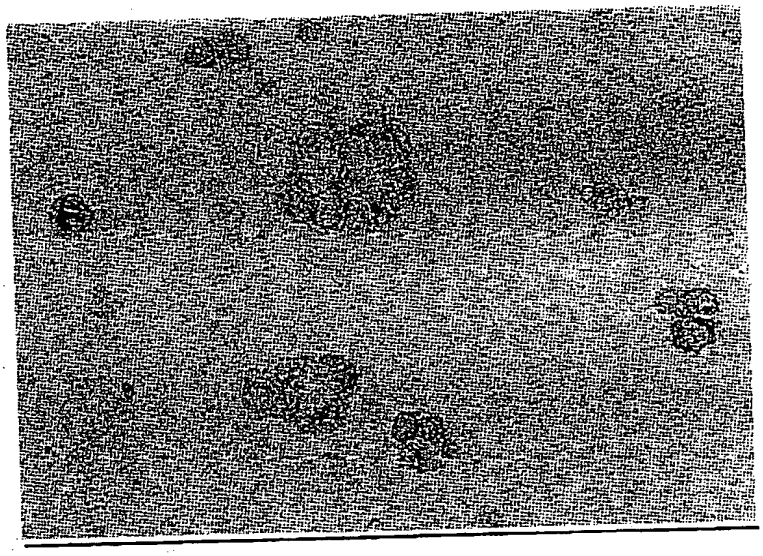
Classically trained in both the physical biochemistry of DNA and microbiology at Princeton and Northwestern Universities and having carried out research on the conformational changes in DNA associated with gene expression prior to the advent of the age of molecular biology in the early 1970's, I was fortunately well positioned to immediately join the exciting molecular biology revolution in the biological sciences. But, from the beginning it was my bias that the most exciting and fruitful studies in this area would be those focused on the molecular aspects of mammalian development. By studying the molecular details of the process of the formation of an organism it seemed reasonable that one would learn much about the processes sustaining life and responsible for defects and diseases of the fully formed organism. Therefore, my laboratory became a unique chimeric mixture of molecular and developmental biology. Staff, graduate students and postdoctoral associates working in the lab are equally able to perform embryonic microsurgery or do complex RT/PCR cloning. This special environment has led to some interesting contributions including the development of the means of producing transgenic animals in 1979, the isolation and maintenance in culture of embryo derived stem cell populations useful in transplantation biology and medicine as well as vehicles for cell-based gene therapy in 1987 and the development of a highly efficient cytoplasmic T7 gene therapy vector in 1993. Since 1998 I have focused all my efforts and those of my Institute (The Oncology Research Institute) on the application of modern molecular and cellular biology to the development of new therapies for the treatment of cancer. The Oncology Research Institute was founded in 1998 with a generous endowment from benefactors and the Greenville Hospital System and the completion of a seven story research tower. My associates and I moved from Ohio to the Institute in late 1998.

The Oncology Research Institute is focused upon reactivating several of the natural barriers to the development of Cancer that exist within the patient. The cells within our bodies are allocated only a certain number of divisions before they undergo programmed cell death, but this natural control mechanism is disrupted in cancer cells. Cancer cells should appear "foreign" to our immune systems and be cleared away by this natural defense mechanism, but somehow cancer cells fail to display their "difference" to our immune systems. As tumors grow they naturally starve themselves for nutrients and would die away for lack of sustenance, were it not for their ability to elicit the development of an independent blood supply. Through the use of new techniques in molecular biology and gene therapy the Oncology Research Institute of GHS is attempting to either (1) "re-engineer" a population of the cancer cells within tumors to display their "foreign" character to the immune system and elicit a strong immune response against the entire tumor as well as distant metastases, (2) reactivate natural cell death signals within cancer cells, or (3) block the formation of tumor blood vessels necessary to sustain tumors. These approaches to the effective treatment of cancer are intended to utilize the unique partnership between cancer scientist and physician that is now a part of the offering of GHS to cancer patients.

LW/9/30/03

Experiment 1

A.



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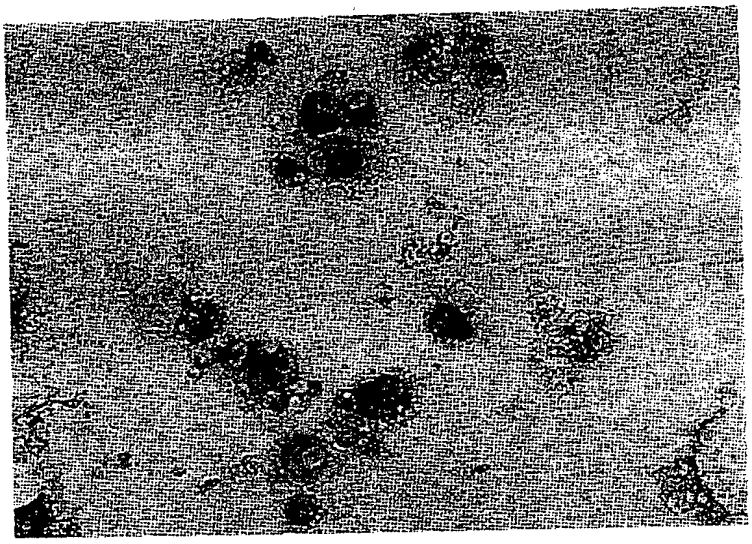
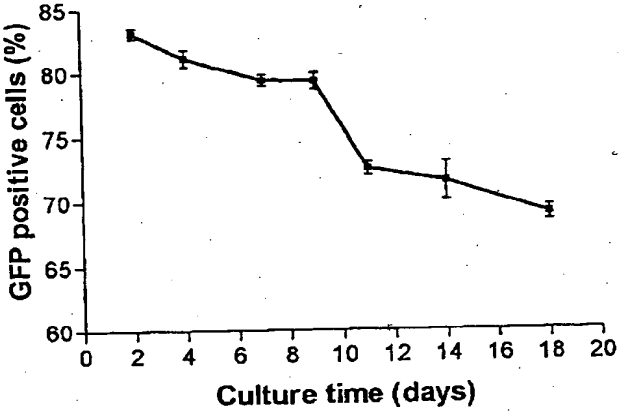


Experiment II

A.



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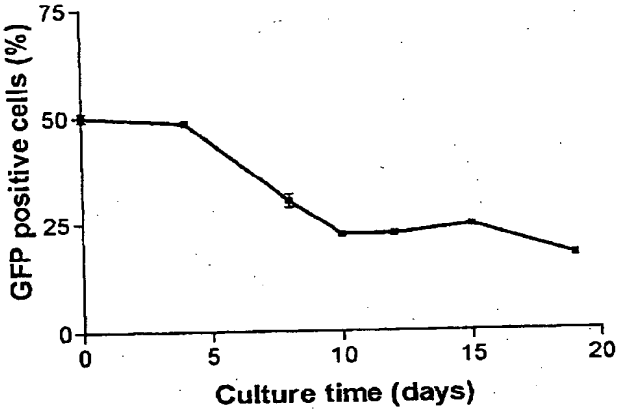
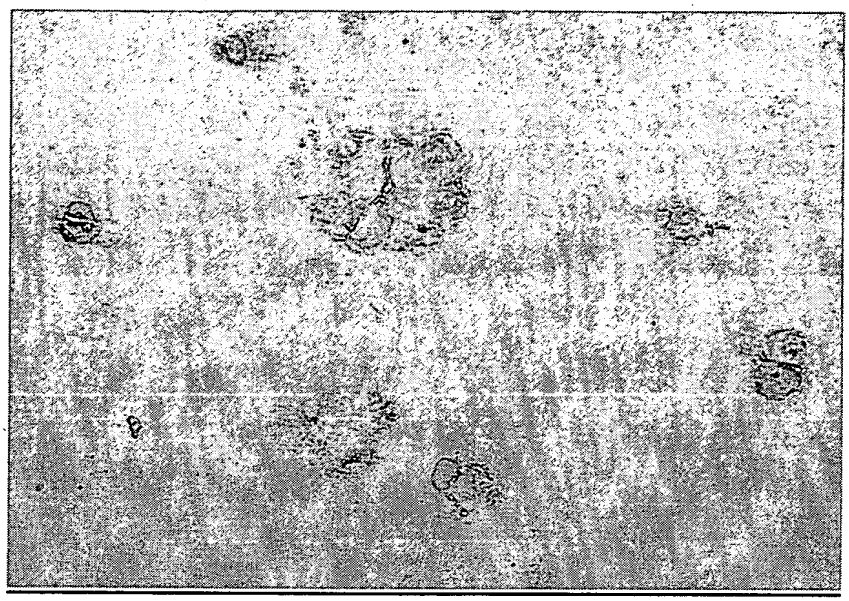


Experiment I

A.



B.